Aspects of the phylogeny, biogeography and taxonomy of galaxioid fishes

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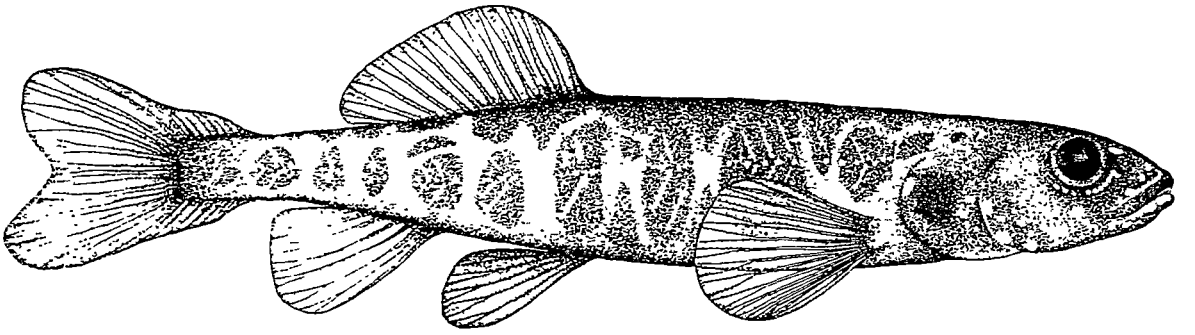

Paragalaxias dissimilis (Regan); illustrated by David Crook

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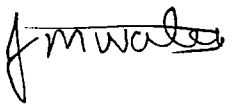
Signed  Date 19 August 1996

Summary

This study used two distinct methods to infer phylogenetic relationships of members of the Galaxioidea. The first approach involved direct sequencing of mitochondrial DNA to produce a molecular phylogeny. Secondly, a thorough osteological study of the galaxiines was the basis of a cladistic analysis to produce a morphological phylogeny.

Phylogenetic analysis of 303 base pairs of mitochondrial cytochrome *b* supported the monophyly of *Neochanna*, *Paragalaxias* and *Galaxiella*. This gene also reinforced recognised groups such as *Galaxias truttaceus-G. auratus* and *G. fasciatus-G. argenteus*. In a previously unrecognised grouping, *Galaxias olidus* and *G. parvus* were united as a sister clade to *Paragalaxias*. In addition, *Nesogalaxias neocaledonicus* and *G. paucispondylus* were included in a clade containing *G. brevipinnis*-like species. A high level of intraspecific diversity was detected between geographic isolates of the South African *G. zebratus* and the widespread *G. maculatus*. The intraspecific divergences are the highest yet reported for fish cytochrome *b*, suggesting that these taxa may represent species complexes.

Phylogenetic analysis of 16S rDNA supported the monophyly of *Paragalaxias*, *Neochanna*, *Galaxiella*, and the *G. truttaceus-G. auratus* group. Again, *G. parvus* and *G. olidus* formed a clade as the sister of *Paragalaxias* and *G. cleaveri* was placed as the sister group of the New Zealand *Neochanna* (a grouping weakly supported in the cytochrome *b* analysis). A large *G. brevipinnis* clade including *Nesogalaxias* was supported, and substantial genetic divergence was detected within *G. maculatus* and *G. zebratus*.

Despite their different evolutionary properties, separate mitochondrial genes produced largely congruent phylogenetic trees, reflecting their common history. Deep phylogenetic splits within the Galaxiinae generally received low bootstrap support in the molecular analyses. Similarly, there was little resolution of galaxioid relationships. This is probably because nucleotide sites that are free to vary become saturated with changes over time. However, one grouping that was weakly supported in both separate molecular analyses received substantial support from the combined molecular analysis. Specifically, the South American *Brachygalaxias bullocki* formed a clade with the Australian *Galaxiella*.

While there have been several morphological studies of the Galaxiinae, until now there has been no cladistic synthesis of these data. In this study, a parsimony analysis of 51 characters in 18 galaxiine species supported some widely accepted groups: *Galaxiella*, *Paragalaxias*, *Neochanna*, and *Galaxias truttaceus-G. auratus*. In addition, the clades [*G. cleaveri*, *Neochanna*] and [*G. zebratus* [*Brachygalaxias*, *Galaxiella*]] received

strong support. Well supported morphological conclusions were corroborated by the molecular and global parsimony analyses, with the exception of the position of *G. zebratus*. The molecular data did not conclusively resolve the position of *G. zebratus*, but weakly supported the above morphological placement.

Galaxiine taxonomy is revised to better represent galaxiine phylogeny, as determined by both morphological and molecular analyses. Specifically, *G. cleaveri* is removed from *Galaxias* and placed in the genus *Neochanna*. Similarly, the three species currently placed in *Galaxiella* are allocated to *Brachygalaxias*. Biogeographical explanations are proposed to account for galaxiine distribution in the light of hypothesised phylogenetic relationships and molecular clock calibrations. The wide distribution of *G. maculatus* is probably due to oceanic dispersal, but the high divergences within this species indicate that its dispersal powers are more limited than previously suggested. The presence of related mudfish on either side of the Tasman Sea is best explained by marine dispersal. Similarly, *Nesogalaxias neocaledonicus* is a descendant of a *G. brevipinnis*-like ancestor that probably colonised New Caledonia in the Pliocene. On the other hand, it is likely that *Galaxiella* and *Brachygalaxias* represent an ancient Gondwanan radiation. It is hypothesised that *G. zebratus* is an ancient Gondwanan ancestor of this clade.

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CHAPTER 1

General Introduction

1.1 Salmoniform intrarelationships

Salmoniform taxonomy is in a state of flux. Many lower salmoniform groupings are well supported and uncontroversial. For example, most workers (Weitzman 1967; McDowall 1969; Fink 1984; Williams 1987; Sanford 1990; Begle 1991; Patterson and Johnson 1995) support a close relationship between the largely Southern Hemisphere galaxioids and the Northern Hemisphere osmeroids. Fink (1984) and Begle (1991) united the two groups to form the Osmeroidei. Similarly, Greenwood and Rosen (1971) and Begle (1992) united the Argentinoidea and Alepocephaloidea to form the argentinoids. Higher salmoniform intrarelationships are the source of considerable confusion. Rosen (1974) limited the Salmoniformes to four groups: esocoids, argentinoids, osmeroids and salmonoids. However, Fink and Weitzman (1982), Fink (1984) and Begle (1992) provided evidence that this assemblage is paraphyletic. Begle (1991) stated that "Salmoniformes" have "become synonomous with the Salmonidae" and suggested that the former term should be abandoned. Subsequently, Patterson and Johnson (1995) removed the esocoids from the salmoniforms. The intrarelationships of these taxa (argentinoids, osmeroids and salmonoids) remain controversial. Sanford (1990) reported an unresolved trichotomy of osmeroids, salmonoids and argentinoids. Fink and Weitzman (1982), Williams (1987) and Begle (1991, 1992) supported a sister-group relationship between the osmeroids and argentinoids, giving a monophyletic Osmerae. In contrast, Patterson and Johnson (1995) concluded, from a study of intermuscular bones and ligaments, that salmonoids are immediately related osmeroids. For the purpose of this thesis, I accept the Osmeroidei of Begle (1991) and Patterson and Johnson (1995), and the Galaxioidea, Galaxiidae and Galaxiinae of Begle (1991).

1.2 The galaxioid fishes

The Osmeroidei (smelts) are made up of two superfamilies: Osmeroidea and Galaxioidea. The largely Southern Hemisphere Galaxioidea of Begle (1991) consists of four families: Salangidae, Retropinnidae, Lepidogalaxiidae, and Galaxiidae.

Family Salangidae

Of the galaxioids, the salangids are anomalous in that they have an entirely Northern Hemisphere distribution. These small translucent fishes occupy the coastal and fresh waters of east Asia. There are six genera containing 14 species, of which 13 are anadromous (McDowall 1987). Salangids are characterised by the presence of an adipose fin, small scales and posterior dorsal and anal fins.

Family Retropinnidae

As defined by Begle (1991), the Retropinnidae comprises three genera: *Retropinna*, *Prototroctes* and *Stokellia*. Retropinnids are characterised by a cucumber odour, an adipose fin, cycloid scales and the absence of a lateral line. The genus *Retropinna* (southern smelts) contains three small anadromous species: *R. tasmanica* (Tasmania), *R. semoni* (Australia) and *R. retropinna* (New Zealand). Landlocked populations of *R. retropinna* may occur sympatrically with anadromous populations of the same species (McDowall 1990). The genus *Prototroctes* (southern graylings) consists of relatively large amphidromous fishes, *P. maraena* (Australia), and a second species *P. oxyrynchus* (New Zealand) which is now extinct (McDowall 1990). Stokell's smelt, *Stokellia anisodon* (New Zealand) is the sole representative of the third retropinnid genus. This anadromous species is found on the east coast of the South Island and spends little time in fresh water.

Family Lepidogalaxiidae

This monotypic family contains the salamander fish *Lepidogalaxias salamandroides*, a strictly freshwater species confined to the south west of Western Australia. It is characterised by scales, long pelvic rays, internal fertilisation and an ability to aestivate (Pusey and Stewart 1989).

Family Galaxiidae

The Galaxiidae comprises *Lovettia*, *Aplochiton* and the Galaxiinae (Galaxiidae *sensu* McDowall 1969). The genus *Lovettia* contains only the Tasmanian whitebait, *L. sealii*. This is an anadromous species with a freshwater stage restricted to the estuarine area of Tasmanian streams and rivers (Fulton 1990). *Lovettia* has a small adipose fin and a lateral line but lacks scales. The dorsal fin is positioned approximately above the pelvic fin. The genus *Aplochiton* contains stout moderate-sized fishes that have a lateral line, adipose fin and a forward dorsal fin (McDowall 1971a). The two species, *A. taeniatus* and *A. zebra*, occur on the Chilean coast of South America. The larger *A. zebra* is also found in the Falkland Islands. This species is characterised by vertical coloration and a relatively deep head. Both aplochitonids are thought to be amphidromous (McDowall 1987). The Galaxiinae currently consists of six genera: *Galaxias*, *Paragalaxias*, *Neochanna*, *Galaxiella*, *Brachygalaxias* and *Nesogalaxias*. These genera contain 40 recognised species, all of which lack both an adipose fin and scales. They are widely distributed in temperate regions of the Southern Hemisphere and are represented on all the Gondwana continents with the exception of Antarctica and India. The Northern Hemisphere description of *Galaxias indicus* from India is considered to be a *nomen dubium* (McDowall 1973a).

Genus *Galaxias*

Galaxias contains all the "typical" galaxiine species. These fishes are characterised by a cylindrical trunk (sometimes compressed) and a dorsal fin that originates above the vent. The genus comprises 28 recognised species and occurs in Tasmania, Australia, New Zealand, South America, South Africa and several islands. A number of the species have a juvenile marine "whitebait" phase. Otolith studies indicate that whitebait remain at sea for from three to over six months (McDowall *et al.* 1994). Diadromous species tend to be large and generalised in character (McDowall 1984). Many members of *Galaxias* can be allocated to distinct species groups, based on morphological observations (e.g. McDowall 1990; McDowall and Frankenberg 1981) and electrophoretic studies (White and Ovenden unpublished). Conversely, the relationships of several species are obscure.

Maculatus group

All members of this group closely resemble *Galaxias maculatus*. They are elongate and slender fishes with a laterally compressed body and small membranous fins. Unlike most galaxiines, these are shoaling species.

G. maculatus

The common jollytail is a medium sized species with an iteroparous life cycle with a high fecundity. It breeds in estuaries and is marginally catadromous (McDowall 1987). The marine whitebait stage of *G. maculatus* may stay at sea for over six months and has been collected 700 km from land (McDowall *et al.* 1975). This species occurs in Tasmania, Australia, New Zealand, South America and also the Chatham Islands, Lord Howe Island, Tierra del Fuego and the Falkland Islands. Landlocked populations are common.

G. rostratus

The Murray jollytail is a medium sized galaxiine known only from the Murray-Darling River system in Australia. Although widespread, its occurrence is intermittent (McDowall and Frankenberg 1981).

G. occidentalis

The western minnow is a medium sized galaxiine that is widespread in the south of Western Australia, inhabiting lakes, streams and swamps. Distinct upland and lowland genetic forms have been found to occur sympatrically, suggesting the presence of reproductive isolation (Watts *et al.* 1995). This species is generally thought to be confined to freshwater but the lowland form may have a marine larval stage.

G. gracilis

The dwarf inanga is a very small galaxiine that occurs in a few lakes along the north west coast of New Zealand's North Island. Its life cycle is spent entirely in freshwater (McDowall 1990).

Truttaceus group

These species closely resemble the first described galaxiine, *G. truttaceus*. They are moderately large, round-bodied fishes; fins are thick and fleshy and usually have dark margins (McDowall and Frankenberg 1981).

G. truttaceus

The spotted galaxias is a large amphidromous species which is common in rivers and coastal lakes around Tasmania and south-eastern Australia. It has also been recorded from a few streams on the south coast of Western Australia (McDowall and Frankenberg 1981). Landlocked populations are known from the central highlands of Tasmania.

G. auratus

The golden galaxias is a large landlocked species found only in Lakes Sorell and Crescent and associated streams in Tasmania's central highlands.

G. tanycephalus

The saddled galaxias is a medium sized species endemic to the Arthurs Lake and Woods Lake region of central Tasmania.

Fasciatus group

This group consists of large, stout-bodied galaxiines with a marine whitebait stage. They are characterised by deep bodies, broad heads and large fins. Juvenile coloration may be similar to that found in young specimens of *G. truttaceus* (McDowall 1984). The three species may occur sympatrically.

G. fasciatus

The banded kokopu is a large amphidromous species found in rivers throughout New Zealand and nearby islands. It is generally a lowland species, but it possesses an ability to climb waterfalls (McDowall 1990). Landlocked populations are known from some lakes.

G. argenteus

The giant kokopu is a very large amphidromous galaxiine that is distributed in drainages throughout New Zealand's North and South Islands; it seldom penetrates far inland.

G. postvectis

The shortjawed kokopu is a large galaxiine that occurs in relatively few but widespread locations throughout New Zealand. This species is found considerable distances from the coast.

Brevipinnis group

This group contains several medium to large-sized species that closely resemble *G. brevipinnis*. They are characterised by a broad but depressed head and a long tubular body.

G. brevipinnis

The climbing galaxias is a large amphidromous species widely distributed in Tasmania and New Zealand. It also occurs on mainland Australia and several islands. It has very large pectoral fins which enable it to climb waterfalls; many landlocked populations are known.

G. fontanus

The Swan galaxias is a moderate-sized galaxiine that is strictly confined to freshwater. It is found only in the upper Swan and Macquarie River systems in eastern Tasmania. Fulton (1978) considered this species to be part of the brevipinnis group and electrophoretic data support this notion (White and Ovenden unpublished).

G. johnstoni

The Clarence galaxias is a moderate-sized galaxiine recorded from two small lagoons in the headwaters of the Derwent drainage in central Tasmania.

G. pedderensis

The Pedder galaxias is a moderate-sized galaxiine confined to the freshwaters of Lake Pedder and associated drainages in southwest Tasmania. Its numbers have fallen dramatically in recent years and it is regarded as endangered.

G. vulgaris

The common river galaxias is a moderate-sized galaxiine present in rivers draining the eastern side of the Southern Alps in New Zealand's South Island. It occurs from just above sea level to altitudes of 300 m. The results of a genetic study by Allibone and Wallis (1993) suggested that *G. vulgaris* is paraphyletic. More recently, isozyme analysis showed that the common river galaxias represents a complex of four genetic types, two of which are sympatric in the Taieri River (Allibone *et al.* 1996). From this complex, McDowall and Wallis (in press) have now described a new species, *G. depressiceps*, and redescribed *G. anomalus*.

Paucispondylus group

This group contains three slender upland species that are confined to fresh water. They are characterised by a short head, small mouth and a long slender caudal peduncle (McDowall 1970).

G. paucispondylus

The alpine galaxias is a moderately small galaxiine that occupies upland streams along the length of New Zealand's South Island.

G. prognathus

The longjawed galaxias is a small species documented from a few alpine streams on either side of New Zealand's Southern Alps. A low elevation population is known from North Otago.

G. divergens

The dwarf galaxias is a small species that occurs in a number of river systems in the north of the South Island and the south of the North Island of New Zealand.

The relationships of the following seven members of the genus *Galaxias* are unclear.

G. cleaveri

The Tasmanian mudfish is a moderate-sized galaxiine that is elongate with small eyes, large nostrils, a rounded caudal fin and strongly developed caudal flanges. It is an amphidromous species (Fulton 1986) that occurs in swampy coastal areas of Tasmania and also Flinders Island and Victoria (Jackson and Davies 1982). The Tasmanian mudfish has an ability to aestivate (Koehn and Raadik 1991) and can tolerate a wide range of temperatures (Andrews 1991).

G. globiceps

This is a moderate-sized galaxiine which has a blunt head, bulbous snout, small fins and is compressed posteriorly. Originally described from Los Alerces in Chile, it was only recently rediscovered from this area (Berra and Ruiz 1994).

G. olidus

The mountain galaxias is a medium-sized species which is rather stocky and has a small head, blunt snout and large mouth. It is widespread in eastern Australia, occurring in coastal drainages and the Murray-Darling system from moderate to very high altitudes. This species exhibits considerable diversity in form, including the distinct geographical types "*fuscus*" and "*findlayi*" which are diagnosed on the basis of colour pattern and morphology (McDowall and Frankenberg 1981). The "*fuscus*" type has been shown to be genetically divergent from other *G. olidus* populations (Rich 1986).

G. parvus

The swamp galaxias is a small stocky species which has a blunt head and small mouth. It occurs only in southwest Tasmania where it inhabits the headwaters of

the Huon and Gordon Rivers. This fish is found in still pools, swamps and streams; the entire life cycle is spent in freshwater.

G. platei

This very large galaxiine is a slender species with a slightly deepened belly. The head is short, broad and strongly flattened. Fins are large and fleshy and there are strong caudal flanges. This species is widely distributed in South America, occurring in Chile, Argentina (either side of the Andes), Tierra del Fuego and the Falkland Islands. The entire life cycle is probably spent in freshwater.

G. rekohua

Mitchell (1995) described this small species from a peat lake in the Chatham Islands. It has a basic *Galaxias* body form and a forked caudal fin. However, it is unusual in having strongly developed caudal flanges, and distinct tubular nostrils.

G. zebratus

The Cape galaxias is a small translucent species that has a fairly stout body, creased lateral line, small fleshy fins and well developed caudal flanges. It occupies flowing or standing waters and is normally found near the heads of pools in small coastal streams (Skelton 1993). The geographic range of this strictly freshwater species extends from the Clanwilliam Olifants system (west coast) to streams on the south coast of South Africa. The known range was recently augmented with the discovery of populations in the Krom and Gamtoos Rivers in the southeast, extending its known range to about 600 km (Cambray *et al.* 1995).

Genus *Paragalaxias*

Paragalaxias contains four small species characterised by forward positioning of the dorsal fin, originating above or slightly behind the pelvic fin. All members are endemic to the Central Plateau of Tasmania and confined to freshwater.

P. eleotroides

The Great Lake paragalaxias is a very small stout-bodied galaxiine which lacks submandibular laterosensory pores. It is present in Great Lake and nearby Shannon Lagoon where it is a bottom dwelling species.

P. dissimilis

The Shannon paragalaxias is a small bullet shaped galaxiine. It occurs sympatrically with *P. eleotroides* in Great Lake and Shannon Lagoon, and is also present in Penstock Lagoon. It may be an active midwater swimming species (McDowall and Fulton 1978).

P. mesotes

The Arthurs paragalaxias is a small species which usually lacks submandibular laterosensory pores. It is endemic to Arthurs and Woods Lakes.

P. julianus

The Western paragalaxias is a moderate-sized species which is endemic to the Western Lakes on the Central Plateau.

Genus *Galaxiella*

Galaxiella contains tiny species that are restricted to freshwater. They are characterised by the presence of longitudinal coloration and a rounded caudal fin. The dorsal fin origin is posterior to the origin of the anal fin and submandibular laterosensory pores are absent (McDowall 1978a). Members of this genus have a one year life cycle and may be able to aestivate (McDowall and Frankenberg 1981).

G. pusilla

The dwarf galaxias is a very small galaxiine that has three black stripes; males have a brilliant orange stripe between the middle and lower black stripes. This species occurs in the streams and lagoons of northeast Tasmania, Flinders Island, Victoria and South Australia. Recently, a new population was recorded from the far northwest of Tasmania (Jackson and Taylor 1994).

G. nigrostriata

The black-stripe minnow is a very small but stocky galaxiine with long gill rakers. Adults have two black stripes separated by an orange band (Berra and Allen 1989). This species is endemic to temporary pools along the south coast of Western Australia.

G. munda

This is a small slender galaxiine which lacks the black stripes present in other members of *Galaxiella*. It is larger than the other two species and is generally found in creeks or pools that are connected to streams (Pen *et al.* 1993). Endemic to Western Australia, it occurs sympatrically with *G. nigrostriata* in the south and also inhabits the west coast.

Genus *Neochanna*

Neochanna contains slender blunt-headed species with small eyes and reduced or absent pelvic fins. In addition, the caudal fin is rounded and caudal flanges extend to the base of the dorsal and anal fins. These fishes are adapted to living in swamps and creeks and have an aestivating ability (Eldon 1978, 1979). It is thought that members of *Neochanna* are restricted to freshwater. Presently this genus is limited to mudfish in New Zealand.

N. burrowsius

The Canterbury mudfish is a medium sized galaxiine with small pelvic fins. It is endemic to slow flowing waters in the Canterbury region of the South Island.

N. apoda

The brown mudfish is a medium sized species that has large flattened teeth (McDowall 1990), a bulbous head and lacks a pelvic fin. It occurs in the southern half of the North Island and the west coast of the South Island.

N. diversus

The black mudfish is a medium sized species that has normal conical teeth and lacks a pelvic fin. It is restricted to swampy regions in the north of the North Island.

Genus *Nesogalaxias*

N. neocaledonicus

The sole member of this tropical genus, the New Caledonian galaxiine is a small species characterised by large eyes and a very long flat head. Pleural ribs are reduced and a deep trunk tapers to a slender caudal peduncle (McDowall 1968). It is endemic to Grand Lac and Lac en Huit in the south of New Caledonia at an altitude of about 250 m. This distribution was recently augmented with the discovery of a new population (Chazeau pers. comm.). The life cycle of this species is completed in freshwater.

Genus *Brachygalaxias*

B. bullocki

This is a tiny galaxiine with a small head, large eyes and a deep trunk. The premaxilla has a long alveolar process and the dorsal fin origin is posterior to the anal fin origin (McDowall 1971b). It is a strictly freshwater species that occurs near the west coast of central Chile. A second Chilean species from near Talca, *B. gothei* (Busse 1982) was synonymised with *B. bullocki* by Berra *et al.* (1995).

1.3 Fossil record

Freshwater fishes are under-represented in the fossil record, largely due to their small, fragile nature (Smith *et al.* 1988). Many ancient fossils of such taxa represent mass death layers in lacustrine rock formations (Wilson 1988). Extant families are typically represented by very few known fossil taxa (Wilson 1992). In keeping with this trend, the galaxiine fossil record is currently limited to a few specimens from New Zealand. Fossilised galaxiines have been found in deposits at Frasers Gully near Kaikorai, Dunedin, and the Foulden Hills near Middlemarch in Otago. McDowall (1976) gathered morphometric and meristic data on these specimens and concluded that the Frasers Gully specimens are *Galaxias brevipinnis* while the Foulden Hills fossils represent *G. vulgaris*. McDowall (1990) consistently states that these fossils date from 7-8 million

years ago (mya), which places them in the Pliocene epoch. His suggestion that they "date from late Oligocene times" (p. 108) is incorrect.

Recently, a large fossilised galaxiine specimen dating from the early Miocene (about 20-25 mya) was discovered in New Zealand. Unfortunately the head of the specimen was not preserved, so conclusions are based on morphometric measurements and estimated vertebral counts (Pole pers. comm.). The single specimen was probably about 380 mm in length, larger than all extant galaxiines with the exception of *G. argenteus*. However, it seems to have been substantially more slender than *G. argenteus*. The fossil was discovered in freshwater sediments and is associated with freshwater mussel and crayfish fossils (Pole pers. comm.).

In addition, fossils of an undescribed galaxioid species have been discovered from a crater lake deposit dating from the Cretaceous, about 70 mya. They are apparently primitive members of the Galaxioidea and show little resemblance to any extant species (Anderson pers. comm.). The specimens are small (maximum 65 mm total length) and seem to have been killed by overturning anoxic water during storm-induced turbidity flows. Some scale remains still show melanophore remnants, but no adipose fin or other membranous tissue is preserved. The specimens lack mesocoracoids, mesopterygoids and palatine teeth. The dorsal fin is located posteriorly, originating just in front of the anal fin. The infraorbitals are curved posteroventrally and the opercle is reduced below the level of hyomandibular articulation (Anderson pers. comm.). All the specimens belong to the South African Museum.

Some salmoniform groups are better represented in the fossil record. The Paleocene osmerid *Speirsaenigma lindoei* (Wilson and Williams 1991) is 30 million years older than any other known smelt. This species provides clear evidence that the osmerids and their relatives were "differentiated long before the middle of the Paleocene" (Wilson and Williams 1991, p. 449). The salmonid fossil record extends back as far as the Eocene (Wilson and Williams 1993). According to Bennett (1984), "fossil evidence indicates that *Thymallus* appeared 60 million years ago, *Salmo* 25 million years ago and *Coregonus* 20 million years ago" (p. 92). These dates may refer to earliest known fossils as listed in Obruchev (1967). However, first fossil records change with new discoveries and are unreliable guides to the age of taxa. The Eocene *Eosalmo driftwoodensis* Wilson (1977) is a stem-group salmonine, probably a precursor of all Recent salmonines. (Wilson and Williams 1993). This suggests, in contrast to the statement of Bennett (1984), that the primitive sister group *Coregonus* arose prior to the Eocene.

1.4 Galaxioid intrarelationships

Rosen (1974) hypothesised a paraphyletic origin for the galaxioids, with retropinnids and prototroctids derived from osmerids in contrast to a salmonid origin for galaxiines and aplochitonids (*sensu* McDowall 1969). However, most systematists support the monophyly of the Southern Hemisphere galaxioid assemblage. Systematic studies of the galaxioid fishes by McDowall (1969), Rosen (1974), Fink (1984), Williams (1987) and Begle (1991) all agree that the genera *Retropinna*, *Stokellia* and *Prototroctes* form a distinct phyletic group. Furthermore, these studies all hypothesise a close relationship between *Lovettia*, *Aplochiton* and the galaxiines. In contrast, the phylogenetic affinities of *Lepidogalaxias* and the Salangidae have been subject to controversy.

Since its description by Mees (1961), the salamander fish *Lepidogalaxias salamandroides* has been difficult to place. It was originally thought to be an unusual scaled member of the Galaxiinae. Frankenberg (1969) examined the osteology of *Lepidogalaxias* and suggested that it is not a galaxiine but the sole member of a closely related superfamily. Rosen (1974) concluded from a study of salmoniform gill arches, caudal skeletons and secondary sexual characteristics that *Lepidogalaxias* is an esocoid. This finding was questioned by Fink and Weitzman (1982) who left the genus unplaced. Fink (1984) argued that although *Lepidogalaxias* shares many reductive characters with galaxiines, it is related to neither the galaxiines nor the esocoids. Rather, he considered *Lepidogalaxias* to be the sister group of neoteleosts. Roberts (1984) noted similarities linking *Lepidogalaxias* with either the galaxiines or galaxioids. From a study of the salmoniform suspensorium and its muscles, Williams (1987) concluded that the Lepidogalaxiidae is the sister group of the Galaxiinae + Aplochitonidae *sensu* McDowall (1969). Begle (1991) conducted a phylogenetic analysis of the osmeroid fishes and agreed that *Lepidogalaxias* is a galaxioid. Johnson and Patterson (1995) were highly critical of Begle's data, suggesting that the majority of his characters were incorrectly coded. However, they agreed that *Lepidogalaxias* is a galaxioid and placed it as the sister group of *Lovettia*.

Similarly, systematists have had difficulty placing the Northern Hemisphere Salangidae. Greenwood *et al.* (1966) included the salangids in the Galaxioidea. However, Weitzman (1967) suggested that, rather than being close to either the galaxioids or osmeroids, the salangids may represent a separate group. McDowall (1969) argued that salangids do not constitute part of the galaxioid radiation, considering them to be a "very specialised offshoot of salmonoids". Fink (1984) supported their placement in the Osmeroidei but was undecided as to whether the salangids formed part of the northern osmeroid or southern galaxioid assemblages. Williams (1987) hypothesised that the Salangidae belongs in the northern osmeroid

radiation. However, a parsimony analysis of both reductive and non-reductive characters (Begle 1991) supported placement of the salangids in the Galaxioidea. Begle suggested that the controversy concerning the placement of salangids stemmed from the highly specialised features of this group. Johnson and Patterson (1995) presented a critique of Begle's analysis and assigned the salangids as the sister group of the osmerid genus *Mallotus*.

1.5 Generic placement of the galaxiines

McDowall (1969) described the generic arrangement of the galaxiines as poorly defined and "in a state of flux". Subsequently McDowall (1970, 1971b, 1972, 1973b, c, 1978a), McDowall and Fulton (1978) and McDowall and Frankenberg (1981) undertook a review of the galaxiine genera. However, it was made clear by McDowall and Frankenberg (1981) that they were unable to reach a consensus on the taxonomic arrangement of the galaxiines; their promised papers discussing galaxiine relationships and biogeography are yet to eventuate.

The New Caledonian galaxiine was originally described as *Galaxias neo-caledonicus* by Weber and De Beaufort (1913). Whitley (1935) placed this species in a separate genus *Nesogalaxias*; this placement was ultimately supported by McDowall (1968). Conversely, the genera *Lyragalaxias* and *Querigalaxias* of Whitley (1935) have never been substantiated. The genus *Austrocobitis* was erected by Ogilby (1899) for *Mesites attenuatus* (=*G. maculatus*). This was supported by Whitley (1935) but rejected by most workers including McDowall (1969, 1970).

There have been several views regarding the generic status of the South African Cape galaxias. Scott (1936) placed *G. zebratus* in the subgenus *Agalaxias*. Stokell (1945) rejected this and subsequently placed it in the Tasmanian genus *Paragalaxias*. Scott (1966) promoted the subgenus *Agalaxias* to a full generic level. McDowall (1969, 1973b) rejected all the above views, regarding *G. zebratus* as a "very ordinary" member of *Galaxias*.

The generic placement of a group of small striped Tasmanian and Australian galaxiines (currently placed in *Galaxiella*) has been controversial. Stokell (1954), Whitley (1960), Scott (1966) and Frankenberg (1969) all accepted an expanded *Brachygalaxias* that included these Australian species in addition to the South American *B. bullocki*. In contrast, McDowall (1970) chose to avoid the supposedly "considerable geographic problems" associated with the occurrence of congeneric freshwater-limited species on separate continents. He argued that similarities between these taxa represented convergent evolution rather than any close phylogenetic relationship. Subsequently,

McDowall (1973c) limited the genus *Brachygalaxias* to include only *B. bullocki*. However, in contrast to his other statements, he suggested that if related, these species (and *Galaxias zebratus*) "may form a very ancient gondwanian relict group". McDowall (1978a) erected the genus *Galaxiella* to contain *G. nigrostriata*, *G. munda* and *G. pusilla*, stating that these species "may be related to *B. bullocki* or...have evolved in a similar direction". Subsequently, McDowall (1984) suggested that inclusion of Australian and South American species in *Brachygalaxias* "confuses the understanding of relationships".

The genus *Neochanna* was originally used to describe New Zealand's brown mudfish *N. apoda* which lacks pelvic fins, a pelvic girdle and mesopterygoidal teeth. The black mudfish *N. diversus* was added to this genus by Stokell (1949). McDowall (1969, 1970) noted the strong similarity between the Canterbury mudfish and the two neochannoids and expanded *Neochanna* to include *N. burrowsius*. The diagnosis of this genus was therefore relaxed to include species with or without pelvic fins and mesopterygoidal teeth.

The genus *Saxilaga* was erected by Scott (1936, 1966) to contain species he considered intermediate between *Galaxias* and *Neochanna*. This genus was diagnosed by characters including the absence of mesopterygoidal teeth and a reduced number of pelvic rays. Included in this genus were the Tasmanian mudfish *Saxilaga anguilliformis* (=*G. cleaveri*), *S. burrowsius* (=*N. burrowsius*) from New Zealand and the South American *G. globiceps*. Andrews (1976) and McDowall and Frankenberg (1981) found that the distinction between *Saxilaga* and *Galaxias* was unsupported due to variable numbers of pelvic rays and mesopterygoidal teeth in *G. cleaveri*. McDowall (1970) stated that the three *Saxilaga* species comprised "similar independent radiations from the central stock of the family", citing the zoogeographical implications of such a widespread group. According to Andrews (1973) the clear similarities between *G. cleaveri* and members of *Neochanna* are "evidently the result of parallel evolution and no direct phyletic relationship...is indicated". He suggested that *G. cleaveri* is morphologically and osteologically most closely related to *G. maculatus*. The discovery of a marine larval stage in *G. cleaveri* (Fulton 1986) shed new light on the dispersal capabilities of this species. Subsequently, McDowall (1990) suggested that the Tasmanian mudfish is "possibly closely related to the New Zealand mudfishes".

1.6 Biogeographical theories

Freshwaters are discontinuous habitats which isolate aquatic taxa in much the same way that islands isolate terrestrial organisms. This heterogeneity, coupled with the generally low carrying capacities of these environments (McDowall 1981), gives rise to rapid

genetic drift and speciation in freshwater fishes. Vicariant events such as mountain uplift and landlocking are major factors likely to disrupt freshwater habitats and lead to speciation. Species lacking salinity tolerance have little potential for dispersal between separate drainages. For movement over large distances, freshwater limited species are reliant on vicariant events such as river capture (Waters *et al.* 1994), landbridge formation (associated with sea level change), and continental drift. In contrast, freshwater species that possess a diadromous life history have greater powers of dispersal.

Of the galaxiines, diadromous species are generally widely distributed. For example the range of *G. maculatus* encompasses Australia, New Zealand and South America; *G. brevipinnis* occurs on both sides of the Tasman Sea. New Zealand's three additional diadromous species are found throughout that country. Conversely, freshwater limited species tend to have restricted distributions. Of Tasmania's 11 non-diadromous species, ten are confined to a single small region. However, some landlocked species are quite widely distributed. For example, *G. platei* and *G. olidus* have geographic ranges of over 1,000 km and occur in many distinct drainage systems. By comparison, the diadromous *G. cleaveri* and *Lovettia sealii* are relatively restricted in their distributions.

The geographic ranges of landlocked species are commonly interpreted in the light of known geological events. For example, Ovenden *et al.* (1993) hypothesised that the ancestors of *G. auratus* and *G. tanycephalus* became landlocked by geomorphological activity within the last 100,000 years (Davies 1974). Landlocked species on either side of Cook Strait and Bass Strait were presumably connected by landbridges during Pleistoceine sea level fluctuations. McDowall (1990) suggested that the Canterbury mudfish *N. burrowsius* was isolated and subsequently speciated as a result of mountain uplift 3-5 million years ago. The biogeographical explanations of such species are not controversial. However there has been no such consensus of workers explaining the wide distribution of diadromous species and the galaxiines as a whole.

McDowall (1970, 1978b) considered diadromy to be a primitive feature of the galaxiines with derived species a result of landlocking. He hypothesised that the galaxiines achieved their current distribution by means of oceanic dispersal. As evidence, McDowall cited the wide geographic range of the diadromous *G. maculatus*, noting the fact that juveniles of this species have been collected up to 700 km from land. McDowall argued that Australia with 20 species is the origin of the group; other areas were sequentially colonised with the aid of the west wind drift. Thus New Zealand (16 species), South America (four species) and South Africa (one species)

represent a "chain of dispersal". This theory is in keeping with the Darwinian "centre of origin" concept.

The increasingly accepted theory of plate tectonics provides an alternative explanation for the austral distribution of galaxiines. Vicariance theorists (Rosen 1974, 1978; Croizat *et al.* 1974; Craw 1979) supported an ancient Gondwanaland origin in place of the dispersal hypothesis. Rosen (1974, 1978) disputed McDowall's claim that the phyletic stability of isolated populations of *G. maculatus* is due to recent dispersal. Instead, he argued that an unusually slow rate of evolution in this species has prevented significant differentiation throughout 70 million years of isolation. Craw (1979) pointed to the similarity of Pliocene fossil galaxiines and Recent species as evidence of such phenotypic stability. Croizat *et al.* (1974) considered dispersal theories to be generally inapplicable in historical biogeography; they rejected the Darwinian "centre of origin" theory. Dispersal theory relies on unlikely events and is not testable while it is claimed that vicariance theory *is* falsifiable. These authors suggested that the only strong evidence for dispersal is seen in taxa with unique distribution patterns. Ball (1975) rejected this notion, but considered McDowall's reasoning to be *ad hoc*.

Vicariance theorists claim that galaxiine distribution can be entirely explained by geological events. They apparently view vicariance and dispersal as incompatible alternatives. McDowall (1990) maintained that dispersal has played a major role in galaxiine biogeography but conceded that the fragmentation of Gondwanaland may have also influenced galaxiine distribution.

1.7 Galaxiine phylogeny

Frankenberg (1969) conducted an evolutionary study of galaxiine species based on external morphology and osteology. In the place of statistical analysis of his data, Frankenberg suggested that Australian members of *Galaxias* comprise five distinct groups:

"olidus" group	*G. olidus*, *G. johnstoni*
"parvus" group	*G. parvus*, *G. pedderensis*
"maculatus" group	*G. maculatus*, *G. rostratus*, *G. occidentalis*
"truttaceus" group	*G. truttaceus*, *G. auratus*
"coxii" group	*G. brevipinnis*

McDowall (1970) conducted a morphological study of the New Zealand galaxiines. From general similarity he suggested some probable species groups and their phylogenetic relationships. McDowall hypothesised that *G. fasciatus*, *G. argenteus*,

and *G. postvectis* are each independently derived from the Australian *G. truttaceus* and thus more closely related to this species than to each other. *Galaxias brevipinnis* and *G. vulgaris* form a group derived from Australian *G. brevipinnis*. Similarly, *G. maculatus* and *G. gracilis* are closely related and possibly derived from Australian *G. maculatus*. *Galaxias paucispondylus* is the primitive member of a group also containing *G. prognathus* and *G. divergens*; McDowall was unsure of the affinities of this group. Finally, the three members of *Neochanna* form a distinct group which may have stemmed from *G. cleaveri*. *Neochanna burrowsius* is the most primitive member of this group.

Several studies have attempted to rigorously establish the relationships of various galaxiine species. These studies focus largely on members of the genus *Galaxias*. Campos (1979) presented a phenetic analysis of 18 galaxiine species based on 32 morphological characters (Fig. 1A); no Australian species were included in this study. Johnson *et al.* (1983) conducted a multivariate analysis of meristic and morphometric measurements from 15 Australian galaxiine species (Fig. 1B). Bennett (1984) combined her own karyological data with that of Johnson *et al.* (1981) to construct a cladogram of some Australian and New Zealand members of *Galaxias* (Fig. 1C). Mitchell and Scott (1979) produced a dendrogram based on muscle myogen proteins of 10 New Zealand species (Fig. 1D). More recently, three allozyme electrophoresis studies have examined the relationships of various species. A study by Barker (1987) used 11 loci to examine nine New Zealand members of *Galaxias* but provided little resolution (Fig. 1E). Allibone and Wallis (1993) examined 13 loci to construct a UPGMA phenogram of five species from New Zealand's South Island (Fig. 1F). White and Ovenden (unpublished) examined 41 loci in a study of 13 galaxiines from Tasmania and one from Australia (Fig. 1G).

1.8 Aims

The lack of congruence between the results of the aforementioned studies suggests that a new approach is required to resolve the relationships of the galaxiines. This thesis will use three methods in an attempt to infer galaxiine phylogeny. The first approach involves direct sequencing of mitochondrial genes to produce a molecular phylogeny. As yet, there are no published nucleotide sequences for this group. Secondly, a thorough osteological study of the galaxiines will be the basis of a cladistic analysis to produce a morphological phylogeny. This will include published morphological data which are yet to be synthesised in any such analysis. Finally, both molecular and morphological data will be combined in an analysis that seeks to use all available evidence (global parsimony). It is thought that historical patterns are best elucidated with such an approach.

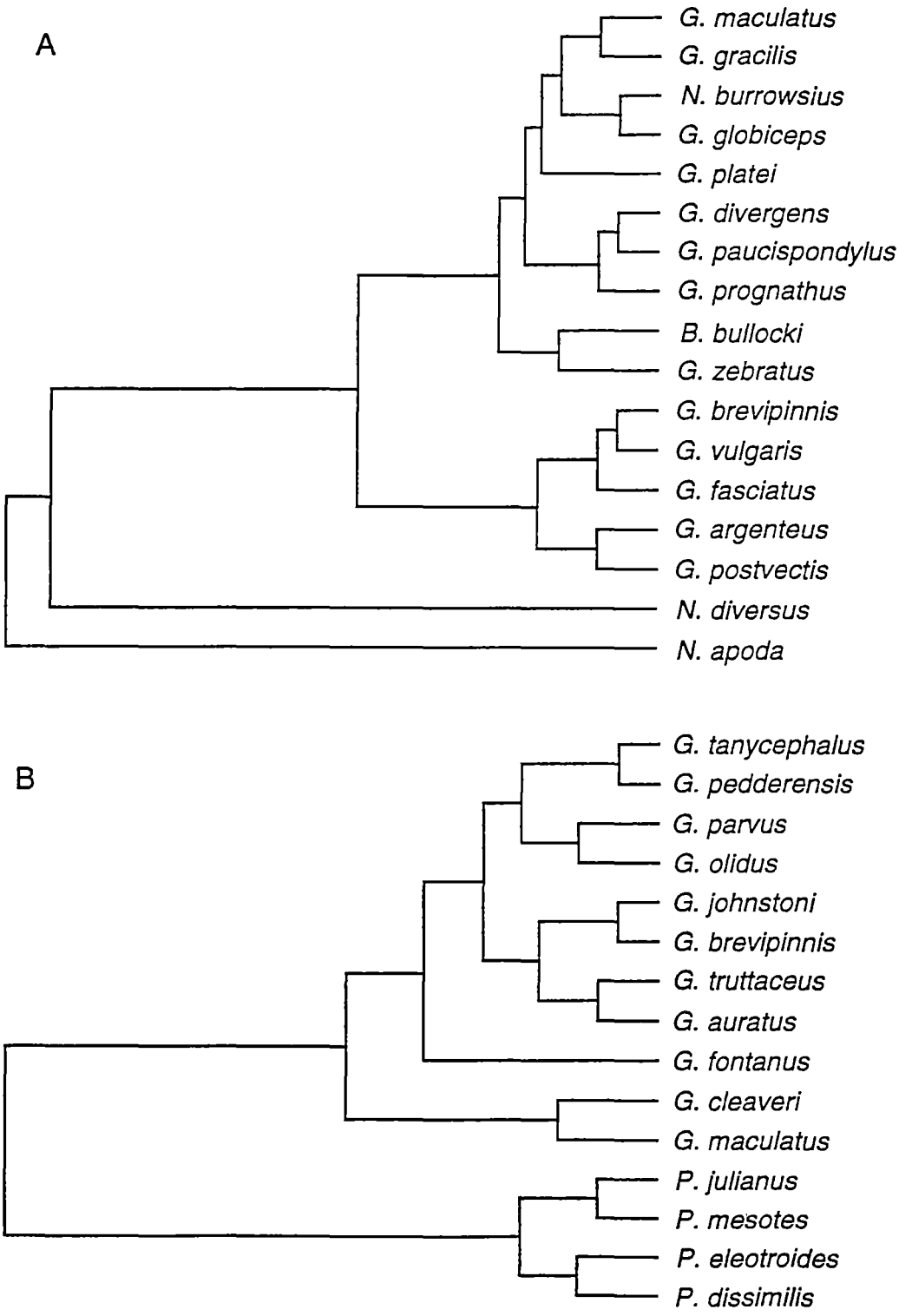

Figure 1. Hypothesised relationships of various galaxiine species, redrawn from (A) Campos 1979; (B) Johnson *et al.* 1983; (C) Bennett 1984; (D) Mitchell and Scott 1979; (E) Barker 1987; (F) Allibone and Wallis 1993; and (G) White and Ovenden unpublished. The phylogeny of Barker 1987 (E) is a parsimony analysis; the other six trees are based on phenetic methods.

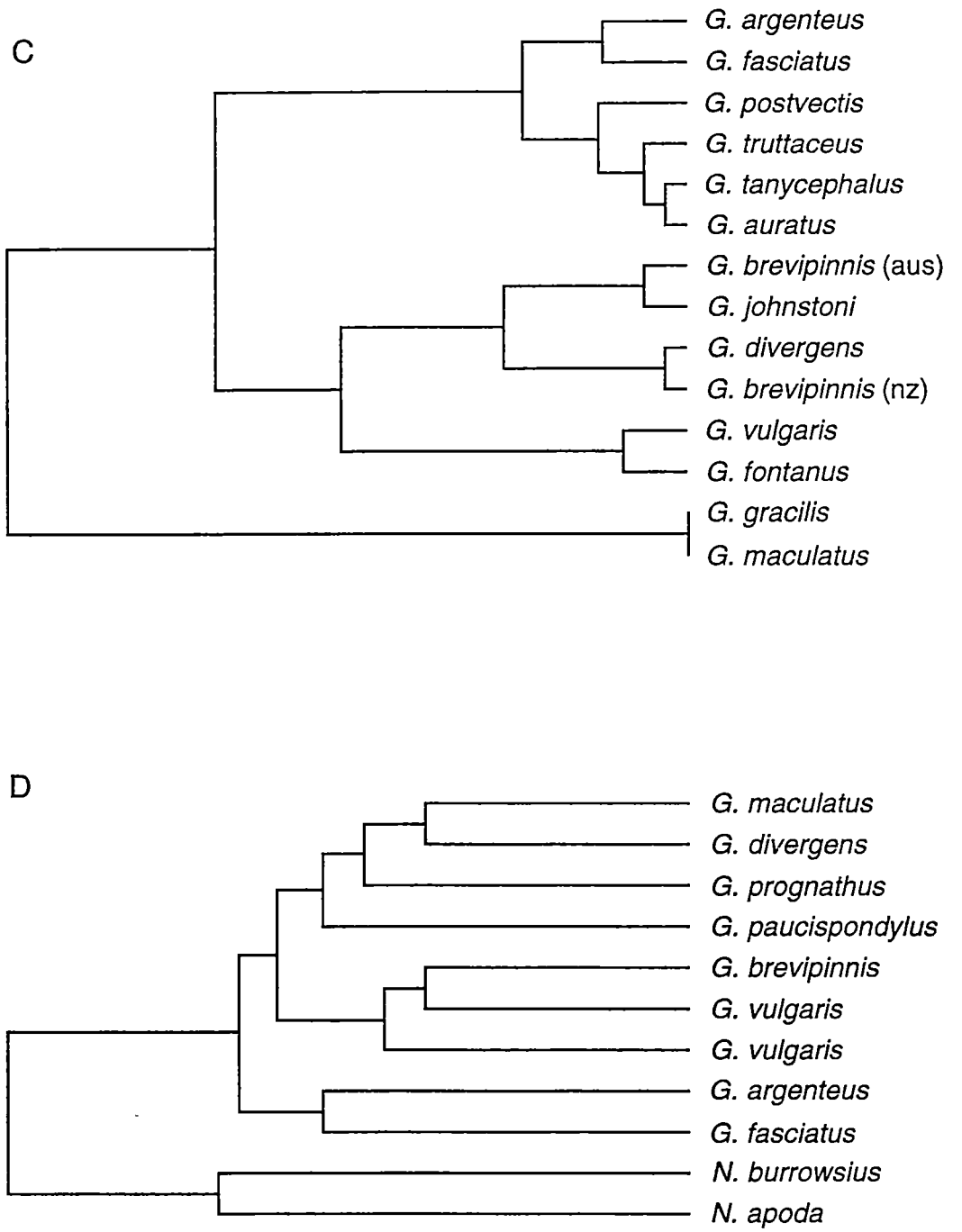

Figure 1.-continued

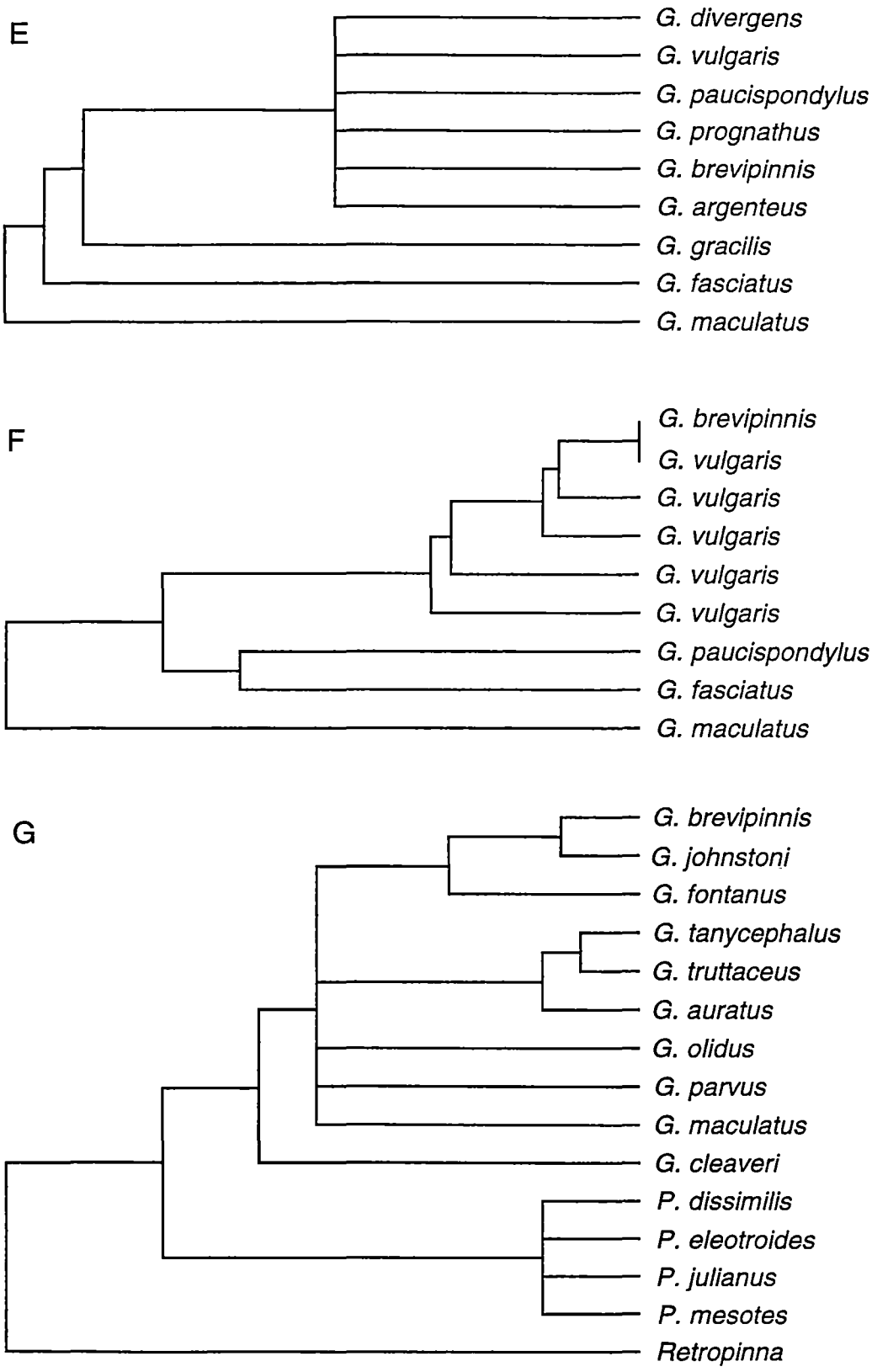

Figure 1.-continued

CHAPTER 2

Molecular Systematics of the Galaxioidea

2.1 Introduction

2.1.1 Mitochondrial genes

The mitochondrial cytochrome *b* gene is a popular tool for phylogenetic studies and has been used to investigate a range of divergence times (Graybeal 1994). Some workers have used this gene to examine organisms which diverged relatively recently, <50 million years ago (mya) while others have attempted to resolve extremely deep phylogenetic branches such as the origin of the tetrapods more than 300 mya (Meyer and Wilson 1990). Most studies indicate that cytochrome b is best suited for the more recent Cenozoic (<65 mya) divergences (Harrison 1989). Mitochondrial protein coding genes generally evolve too rapidly to provide reliable information on ancient divergences (Hillis and Huelsenbeck 1992; Graybeal 1994). Graybeal (1993) examined the phylogenetic utility of this gene in inferring bufonid frog phylogeny. She concluded that in bufonids cytochrome *b* gene evolution is strongly constrained by selective forces, more so than in other vertebrates. There is increasing evidence that rates of sequence evolution vary between groups of taxa, possibly correlated with metabolic rate (Martin and Palumbi 1993). For instance, rates of cytochrome *b* nucleotide substitution in sharks appear to be up to eight times slower than in mammals (Martin *et al.* 1992). Thus the phylogenetic utility of the cytochrome *b* can not always be predicted *a priori.*

Mitochondrial rRNA genes evolve considerably faster than their nuclear counterparts (Mindell and Honeycutt 1990). This may be due to decreased polymerase efficiency or reduced efficiency of repair mechanisms (Holland *et al.* 1982). Mindell and Honeycutt (1990) suggested that the mitochondrial 16S rRNA gene may be useful for resolving divergences that occurred 150 mya (and possibly as long as 300 mya). However, as with the cytochrome *b* gene, most studies indicate that 16S rDNA is particularly useful for examining Cenozoic divergences (Hillis and Dixon 1991). Beyond a certain level of divergence, multiple substitutions occur at a given site. The subsequent homoplasy confounds phylogenetic analysis. However, the time scale at which this occurs is dependent on the nucleotide substitution rate of the taxonomic group of interest.

Unlike nuclear genes which recombine and are biparentally inherited, the mitochondrial genome is inherited maternally as a single locus. Therefore, separate mitochondrial genes are connected to a single sequence phylogeny (Miyamoto *et al.* 1994). That is not to say that the mitochondrial DNA (mtDNA) tree is necessarily congruent with the species tree. There are some factors which may cause this to be not the case, such as hybridisation (see below). However, in spite of their different evolutionary properties,

separate mtDNA genes should produce congruent trees, reflecting their common history. In cases where mtDNA gene trees are not wholly congruent, topological differences may reflect inadequate information rather than true conflict between the data sets. Analysis of near-most-parsimonious trees should determine whether this is the case (Swofford and Olsen 1990).

Alternatively, lack of congruence may stem from systematic error inherent in the phylogenetic analysis. Compositional bias may systematically distort character state reconstruction (Collins *et al.* 1994). For example, taxa that have independently evolved similar G+C base frequencies may be incorrectly grouped together (Steel *et al.* 1993). Similarly, the presence of long unbranched lineages may artificially influence tree topology. Long branches are often characterised by many homoplasious changes that are not detected as such (Felsenstein 1978). Unbranched lineages tend to exhibit chance convergence and are often linked by parsimony analysis. In this way a distant outgroup will generally join the longest branch of the ingroup, giving an unreliable topology (Wheeler 1990). Such systematic error can be partially avoided by weighting characters to emphasise rare substitutions, thereby reducing levels of homoplasy.

2.1.2 Character weighting and homoplasy

A tree derived from weighted data should emphasize the best available evidence and thus be a more reliable estimate of species phylogeny than one from unweighted data (Cracraft and Helm-Bychowski 1991). In accordance with this, Fitch and Ye (1991) observed that weighting improves the performance of phylogenetic analysis. Before assessing which characters are likely to exhibit homoplasy, it is important to understand the properties of the gene in question. The base sequence of cytochrome *b* and other protein-coding genes corresponds to the amino acid sequence of their polypeptide products. Amino acids are coded by groups of three bases (codons). Of the 64 possible codons, 61 encode particular amino acids while the other three terminate chain synthesis (Stryer 1988). A natural division for character weighting in protein-coding genes is based on codon structure. Codons in which nucleotide changes cause amino acid substitution can be preferentially weighted. Alternatively, homoplasy may be reduced by weighting positions within a codon. Most of the 20 naturally occurring amino acids are encoded by more than one codon. Changes at third codon positions are usually called silent because they do not cause a change in the encoded amino acid sequence. First and second codon positions determine amino acid composition and are heavily constrained by selective forces. Substitutions at first and second positions occur considerably less frequently than silent changes (Kocher *et al.* 1989) and may be preferentially weighted.

Mitochondrial 16S rDNA is subject to strong selective constraints, with as few as 10% of the nucleotide positions free to vary (Xiong and Kocher 1993). To function properly in a ribosome, rRNA folds into a secondary (2°) structure which depends on the primary gene sequence (Noller and Woese 1981). Ribosomal RNA genes consist of unpaired loop regions and paired stem regions. Estimates of 2° structure are generally based on comparative analysis of aligned sequences (Schnare *et al.* 1996). Ribosomal RNA sequences support an endosymbiotic eubacterial origin for mitochondria (Gray *et al.* 1989). Thus the mitochondrial 16S rRNA gene of eukaryotes is analogous to the 23S rRNA gene of prokaryotes. The arrangement of stems and loops in the 16S (23S-like) rRNA is similar across a range of eukaryotic organisms, despite evolution of the primary sequence (Gutell *et al.* 1993). This stability is due to compensatory changes that occur between paired nucleotides in stem regions (Torres *et al.* 1990). As noted by Xiong and Kocher (1993), many workers preferentially weight stem regions over loop regions as the former are expected to be more conserved (e.g. Miyamoto *et al.* 1994). However, Noller and Woese (1981) noted that in 16S rRNA "functionally important sites appear to be located in unpaired regions and are phylogenetically highly conserved". Wheeler and Honeycutt (1988) argued that the constraints of 2° structure make stem regions unreliable for phylogenetic studies. They recommended that paired regions be eliminated or assigned one half weight to compensate for their nonindependence. Hillis and Dixon (1991) suggested that weighting of paired versus unpaired bases should be close to one and derive from observed levels of compensation. A few workers have chosen to restrict differential weighting to within stem regions. Caccone *et al.* (1994) assigned compensatory changes (those that maintain Watson-Crick base pairing) one half the weight of non-compensatory changes.

Another means of extracting phylogenetic signal from molecular data involves differential weighting of particular types of character change, irrespective of the character's position (codon position, stem/loop). Of the possible base substitutions, transitions (purine-purine or pyrimidine-pyrimidine) occur far more frequently than transversions (purine-pyrimidine or pyrimidine-purine). This transition bias is particularly evident in the mtDNA of some vertebrate taxa (Brown *et al.* 1982; Hillis *et al.* 1993). Vertebrate sequences may may be saturated with transitions after only 10-20 million years of divergence. The homoplasy associated with transition bias may be partly overcome by preferentially weighting transversions. Indeed, some studies entirely eliminate transitions from phylogenetic analyses (Bowen *et al.* 1993; Avise *et al.* 1994). However, Kraus and Miyamoto (1991) found that analysis of transversions alone resulted in the loss of information at lower hierarchical levels. Most studies include both transitions and transversions, often with preferential weighting of the

latter. Some workers have attempted to overcome substitution bias (due to compositional bias) by weighting different categories of transitions and transversions according to their expected to observed ratio (EOR; Knight and Mindell 1993).

2.1.3 Sequence alignment and gap weighting

Ribosomal RNA genes lack the selective constraints associated with codon structure. As a result, nucleotide insertions and deletions are relatively common and give rise to length variation in rDNA sequences. Meyer (1994) noted that length variation makes 16S sequences for distantly related species difficult to align. Swofford and Olsen (1990) suggested that "when regions of sequence are so divergent that a reasonable alignment cannot be attained by manual methods...those regions should probably be eliminated from the analysis". While this strategy requires the worker to subjectively discard data, they argued that "the researcher has already discarded data, in a sense, by choosing to sequence one molecule rather than another". Similarly, Meyer (1994) suggests that questionably aligned sequences are best excluded from analyses.

Miyamoto and Cracraft (1991) noted that tree topology is heavily influenced by the choice of alignment. Many workers use computational alignment methods to insert gaps between sequences. However, the addition order of taxa may affect the final alignment. For example, results will be misleading if sequences from distant relatives are aligned before sequences of closely related species. There is an element of circularity required to provide realistic sequence alignments. Mindell (1991) argued that a knowledge of phylogeny is required to choose between different alignments. He noted that "few systematists would seek to determine character homology without considering hypothesised relationships among taxa." Many workers use such hypothesised relationships to correct "mistakes" in computer-generated alignments.

Weighting of gaps in phylogenetic analysis is problematic. Some workers choose to treat insertions and deletions as missing data (e.g. Knight and Mindell 1993; Gillespie *et al.* 1994). This strategy minimises the impact that spurious alignments can have on tree topology. Alternatively, some systematists implement the arbitrary gap costs that were initially used to generate an alignment (e.g. Wheeler 1995). If these gap weights are heavy, tree topology may be largely determined by the choice of alignment. Clades produced by this method may receive strong statistical support, but this does not necessarily indicate that they represent the true phylogeny.

2.1.4 Congruence between separate mtDNA data sets

The reliability of alternative weighting strategies can be assessed by measuring congruence between separate mtDNA gene phylogenies (Miyamoto *et al.* 1994). The

preferred weighting scheme is the one that gives the greatest level of congruence. There are two contrasting approaches to the measurement of congruence between separate data sets: taxonomic congruence and character congruence. The first method compares cladograms by means of consensus. There are several consensus techniques which summarise cladogram information. The simplest and most conservative approach is a strict consensus tree (Sokal and Rohlf 1981), in which only the groups common to all cladograms are supported. Resolution can be increased by expanding the level of consensus to include unresolved, non-contradictory groups. Bremer (1990) favoured this approach, calling it the combinable component consensus. Nelson trees (Nelson 1979), Adams trees (Adams 1972) and majority-rule consensus trees (Margush and McMorris 1981) all contain more information and are more fully resolved. However, these methods contain components that are not supported by at least one of the mp cladograms.

An alternative measure of congruence, character congruence, assesses character conflict between data sets. Miyamoto *et al.* (1994) examined congruence between cladograms from three separate mitochondrial genes. For each gene they calculated the number of extra steps (relative to mp trees) required by each possible dichotomous topology. The number of extra steps was adjusted to account for the number of informative sites present. Their study included five species, with only 15 possible pairwise groupings. For larger numbers of taxa, alternative measures of character congruence are required. Mickevich and Johnson (1976) used two methods to assess the congruence of their data sets. One method involved measuring the fit of each set of characters to the tree computed from the combined data set, similar to the approach of Miyamoto *et al.* (1994). The second method compares the tree for each data set to that for the combined set. Mickevich and Farris (1981) favoured the latter method, assessing congruence by measuring the number of extra steps that result from combining the data sets. Simply, this method measures [Treelength AB - (Treelength A + Treelength B)] where A and B are separate data sets. The number of extra steps decreases with increasing character congruence (Wheeler 1995).

2.1.5 Incorrect phylogenetic inferences from molecular data

Nucleic acid sequences generally provide useful information from which to infer phylogeny. However, some phenomena may give rise to misleading molecular tree topologies. Such factors include paralogy, stochastic lineage sorting and introgression. In contrast to mtDNA, nuclear genes may be paralogous, *i.e.*, duplicated in separate parts of the genome, or form multigene complexes. A multigene complex is a group of homologous genes with similar functions. For example, some plants have genes encoding glutamine synthetase isozymes that are expressed in both the chloroplasts and

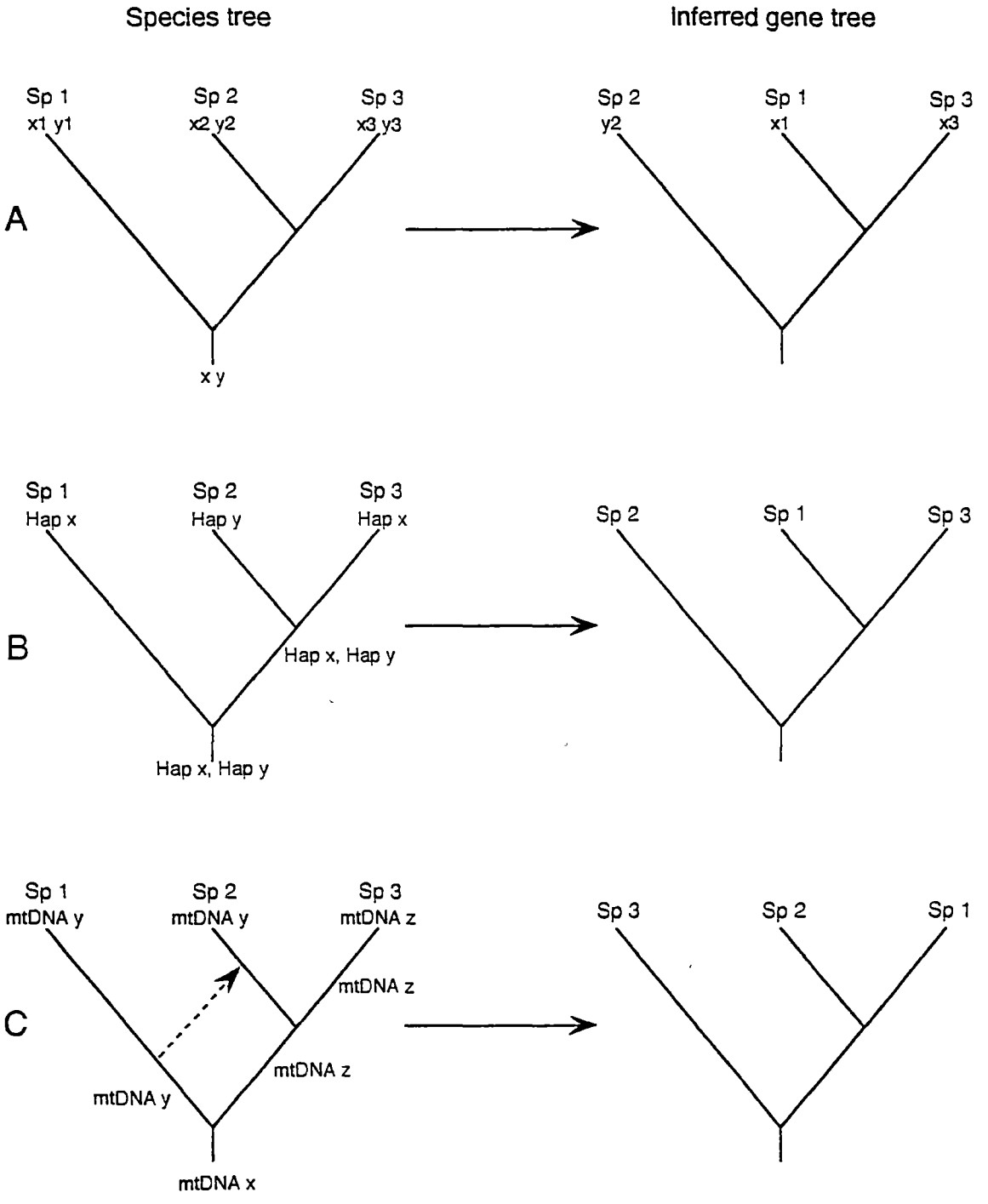

Fig. 2.1 Hypothetical cases in which molecular data lead to incorrect phylogenetic inferences. A. Paralogy. The worker is unaware that duplicated genes (x and y) are present and samples only x1, y2 and x3. The incorrect inference is that species 1 and 3 are sisters After Doyle (1992). B. Stochastic lineage sorting. The random extinction of an ancestral polymorphism, involving alleles (haplotypes) x and y, incorrectly links species 1 and 3. After Doyle (1992). C. Hybridisation. The introgressive transfer of mtDNA y into species 2 incorrectly implies that species 1 and 2 are sisters. After Moore (1995).

the cytosol (Edwards *et al.* 1990). Such genes can be used to accurately infer phylogenetic relationships. However, if the the presence of multiple copies is not recognised, or expression is lost through evolution, the inferred phylogeny may be incorrect (Doyle 1992; see Fig 2.1A).

Alternatively, lineage sorting may confound molecular analyses. Under genetic drift, there is a possibility of obtaining spurious phylogenetic information due to the segregation of ancient polymorphisms (Fig. 2.1B). Even if a gene tree is unambiguous, the species tree may be quite different (Wu 1991; Doyle 1992). Many species are characterised by a considerable amount of genetic diversity (polymorphism). Variation is typically present in both nuclear and mitochondrial genes. For example, Ovenden and White (1990) found 58 distinct mtDNA lineages (haplotypes) to be present in 150 specimens of riverine *Galaxias truttaceus*. Where polymorphisms are ancestral, mtDNA variants often occur across species boundaries (Avise *et al.* 1987). Similarly, allozyme studies show that polymorphisms are commonly shared by species within clades (e.g. Ovenden *et al.* 1993; Allibone *et al.* 1993). Events such as population bottlenecks and founder events may increase the rate of stochastic lineage sorting and extinction. The resultant likelihood of incorrect phylogenetic inference depends on levels of polymorphism at the time of speciation, and increases if bifurcations occur in rapid succession (Wu 1991). However, in contrast to nuclear gene phylogenies, mtDNA trees are seldom confounded by lineage sorting. As mtDNA is maternally inherited and effectively haploid, its effective population size is one quarter that of a nuclear-autosomal gene. When internodes are short, an mtDNA tree has a much greater chance of showing the correct topology than does a nuclear gene tree (Moore 1995).

Finally, introgression may lead to incorrect phylogenetic conclusions. Hybridisation may give rise to the flow of nuclear and mitochondrial genes across species boundaries (introgression). Hybrids can be detected as such by the presence of morphological and nuclear gene (e.g. allozyme) data that are intermediate between two species. While the effects of nuclear introgression become diluted over time through recombination, the effects of mtDNA introgression may be preserved through maternal lineages (Bernatchez *et al.* 1995). Introgressive transfer of mtDNA requires hybrid fertility and repeated backcrossing in successive generations of female descendants to males of the paternal species (Campton 1990). A clear case of introgression was documented in European mice by Ferris *et al.* (1983) when the mtDNA of one species was found in "pure" members of another species, well away from the known hybrid zone.

Hybridisation occurs naturally in fish more commonly than in other vertebrate groups (Campton 1987). A number of factors may contribute to this trend. These include external fertilization, competition for limited spawning habitat, and the likelihood of secondary contact between recently evolved forms. Complete interspecific replacement of mtDNA has been reported in members of the cyprinid genus *Notropis* by Dowling and Hoeh (1991). Similarly, Bernatchez *et al.* (1995) reported a population of brook char (*Salvelinus fontinalis*) in which every individual sampled had mtDNA characteristic of Arctic char (*S. alpinus*). This was despite the absence of Arctic char from the drainage occupied by the population of brook char. In this case, the absence of the donor species suggests ancient rather than recent introgression.

Introgression is potentially a serious problem for mtDNA phylogenies. Once introgression occurs, the mtDNA gene tree may conflict with the tree of the species involved (Doyle 1992; see Fig. 2.1C). While recent introgression can be detected with morphological and nuclear genetic data, ancient introgression events may be difficult to recognise as such. Possibly the strongest evidence for ancient introgression is incongruence between the basal groups of well resolved mtDNA and nuclear gene/morphological phylogenies (Moore 1995). However, it is worth noting that hybridisation is far more likely to occur between sister taxa than between distantly related species. In such cases, ancient introgression may not change tree topology. Rather, bifurcations will appear to have occurred more recently than their true date.

2.1.6 Phylogeny reconstruction

Some methods of phylogeny reconstruction involve the analysis of discrete characters. With this approach, a character state is assigned to each taxon for each character. For molecular data, each nucleotide position represents a character, with the four bases G, A, T or C representing the possible character states. The systematist may choose to treat gaps as a fifth character state. At a nucleotide position, a base may be replaced by any one of the other three bases, so DNA sequence characters are normally treated as unordered (Avise 1994). Discrete character methods include cladistics and maximium parsimony analysis. The cladistic approach of Hennig (1966) relies on the presence of synapomorphies. That is, the phylogenetic relationships of taxa are deduced by their shared-derived character states. In theory, a clade can be diagnosed by a single synapomorphy. However, to have confidence that a clade is real rather than an artefact of convergence, a large number of characters must be sampled. It could be argued that DNA sequences are poorly suited to Hennigian cladistic analysis because of the high likelihood of homoplasy (reversals and parallelisms). Character conflicts resulting from such homoplasies may be addressed by the use of maximum parsimony (Felsenstein 1983). Maximum parsimony analysis is based on the conservative assumption that

evolution proceeds via fewer rather than a greater number of steps. That is, no more evolutionary changes are assumed than are necessary to explain the distribution of character states among species. From calculations of character state changes on alternative trees, maximum parsimony favours the topology with the smallest number of character state changes (Fitch 1971). This approach seeks to minimise the number of homoplasies. Maximum parsimony analysis has several advantages over other methods, including the sophistication of parsimony algorithms and the lack of assumptions about sequence evolution (Cracraft and Helm-Bychowski 1991).

Like parsimony analysis, the maximum likelihood (ML) method uses discrete characters to reconstruct phylogenies. However, while parsimony methods make minimal assumptions, the ML technique requires the specification of a predetermined evolutionary model. With each model, nucleotide positions are considered to evolve independently (Felsenstein 1981). The Jukes-Cantor model assumes that base frequencies are equal and incorporates equal probabilities of substitutional change (Saitou 1990). The Kimura two-parameter model accommodates different substitution rates for transitions and transversions. A third method, the generalised two-parameter model encompasses both previous models. This model accommodates unequal base frequencies but assumes that they are at equilibrium. The generalised two-parameter model is the most realistic of the three. For the ML method, tree likelihood is computed by calculating the likelihood of each base occurring at each node as a function of branch lengths and branch order (Swofford and Olsen 1990). The relative likelihood of all possible tree topologies is determined. The favoured topology is the one with the highest likelihood (Fukami and Tateno 1989). The ML approach is potentially powerful and reliable. The major drawback of this method is computational difficulty, which increases with more taxa and also as models of sequence evolution become more realistic.

Alternatively, phylogenies may be reconstructed using distance methods. Unlike column by column analysis of discrete characters, distance (phenetic) methods examine overall similarity and construct a distance matrix (Doolittle and Feng 1990). The matrix consists of estimated pairwise genetic distances between taxa (Avise 1994). The similarity of two sequences can be measured simply as the number of nucleotide positions containing matching bases divided by positions containing non-matching bases. A drawback of the distance approach involves the accumulation of multiple substitutions over time, at a given site. Distance increases rapidly at first, but the rate of increase plateaus as sequences become saturated with substitutions. However, the problem of multiple substitutions may be overcome by making assumptions about sequence evolution and applying correction factors that correspond with evolutionary

intervals (Swofford and Olsen 1990). Commonly used correction models include the two parameter model of Kimura (1980) and the maximium likelihood model of Felsenstein (1981). Another potential problem with distance methods is the construction of trees from pairwise distances (percentages) rather than from the DNA sequences themselves. This represents the loss of information. For example, Penny (1982) showed that different sets of DNA sequences may yield identical distance matrices. However, Swofford and Olsen (1990) suggested that information loss is not a serious problem when dealing with real data. There are several algorithms commonly used to reconstruct phylogenetic trees from distance matrices. The neighbour-joining method (Saitou and Nei 1987) is a stepwise method that allows for negative branch lengths and does not assume an equal rate of evolution for all taxa. This algorithm adjusts branch lengths between pairs of nodes by calculating the mean divergence from all other nodes. Similarly, the algorithm of Fitch and Margoliash (1967) allows for non-uniform rates of evolution. This method compares the branch lengths of all possible trees. Alternatively, trees can be constructed by cluster analysis. The UPGMA of Sneath and Sokal (1973) is a commonly used clustering algorithm. Initially, the two taxa with the smallest pairwise distance are grouped. Additional clusters are determined on the basis of (arithmetic) mean distances between taxa. This method is based on the simple but stringent assumption that the rate of sequence evolution is homogeneous.

2.1.7 Assessing phylogenetic confidence

The bootstrap of Felsenstein (1985) is a confidence test that resamples the original data set. Bootstrapping resamples with replacement to create multiple replicate data sets of the same size as the original data matrix. Each replicate data set is analysed independently. Consensus techniques can then be used to determine the percentage of replicates in which a particular clade is supported. This percentage is used as a measure of statistical support for that clade. According to Felsenstein (1985), "If a group shows up 95% of the time or more, the evidence for it is taken to be statistically significant". The underlying assumption of the resampling technique is that characters evolve independently and are independently chosen by the worker. Both of these assumptions are frequently violated by both molecular and morphological data sets. Nevertheless, many systematists rely on the bootstrap as the sole test of confidence for their phylogenetic studies. Some workers argue that statistical confidence should or can not be associated with branches on a phylogenetic tree. For example, Carpenter (1992) noted that normal statistical methods require a probability distribution for the character universe that is sampled. He suggested that such a distribution is impossible to construct for phylogenetic analysis. Sanderson (1995) countered this by constructing such a probability space for a simple molecular data matrix.

There is some controversy over the utility of bootstrapping as a technique, suggesting that further study is required (Li and Zharkikh 1994). Hillis and Bull (1993) conducted a simulation study and concluded that bootstrap values over 50% consistently underestimate phylogenetic accuracy. They found that, under some circumstances, the vast majority (>95%) of clades with bootstrap estimates over 70% were correct. Furthermore, bootstrap estimates vary with the addition of new taxa and new characters, especially when the number of taxa is small. Thus values are not comparable between studies. They noted that "many workers treat bootstrap results as statements about the probability that a particular group is a real historical group". In fact, Hillis and Bull (1993) stated that bootstrapping "should provide an indication only of the degree of support of a particular technique for a clade". Felsenstein and Kishino (1993) do not dispute these findings. However, they suggested that "suspending all belief in the *P* value except as a measure of relative confidence...would be an over-reaction". Instead, they proposed an alternative interpretation of bootstrap values. Where *P* is a bootstrap estimate for a clade, 1 - *P* is "a conservative assessment of the probability of getting that much evidence favouring the group if it is not present".

Most molecular systematists continue to use bootstrap estimates, but interpret them as conservative estimates of phylogenetic accuracy (e.g. Gillespie *et al.* 1994). Results are frequently presented as a majority rule bootstrap consensus tree (e.g. Milinkovitch *et al.* 1993). Workers generally regard bootstrap values of over 50% as indicating support for a clade while estimates below 50% give weakly supported, tentative conclusions (e.g. Ballard *et al.* 1992; Knight *et al.* 1993). Reported bootstrap values appear to correlate well with other measures of confidence (Linder 1991; Cracraft and Helm-Bychowski 1991). Li and Gouy (1991) noted that many studies repeat the resampling process only 100 times. They recommended the use of several hundred bootstrap replicates, especially when many species are involved.

Confidence in a particular clade may also be assessed by examining near-most-parsimonious (nmp) trees (Cracraft and Helm-Bychowski 1991). If a clade is present in the shortest tree, and all trees within a few steps of the shortest tree, then more confidence can be placed in that grouping (Swofford and Begle 1993). Several studies have illustrated the relative robustness of clades by indicating the number of extra steps required before they are not supported by strict consensus (e.g. DeSalle 1992; Hong *et al.* 1993; Schneider *et al.* 1993). Such values may be termed "decay indices" as in Savolainen *et al.* (1994), or Bremer support values as in Brower (1996). Thus, those sections of the tree that are strongly supported are distinguished from sections which are less robust. Alternatively, Cracraft and Helm-Bychowski (1991) reported nmp results as a majority rule consensus of all trees within five steps of the mp tree, and as a

majority rule consensus of all trees within 1% of the length of the mp tree. They found that the former method provided strong resolution for recent clades while the latter method did not. Unfortunately, analysis of near-optimal trees is restricted to discrete character methods. While bootstrapping can be applied to phylogenies constructed from both parsimony and distance methods, most distance algorithms lack the ability to examine large numbers of suboptimal trees. Moreover, computational limitations make bootstrapping impractical for the maximum likelihood method. Instead, a test is employed to assess whether the preferred (ML) tree is significantly more likely than others.

2.2 Materials and Methods

2.2.1 PCR with universal primers

The polymerase chain reaction (PCR), developed by Mullis and Faloona (1987) and Saiki *et al.* (1988), is an enzymatic cloning technique used to amplify specific DNA sequences. In this method, two synthetic oligonucleotide primers hybridise to opposite strands and flank a targeted section of DNA. A series of temperature cycles involving denaturation, primer annealing and primer extension gives rise to exponential amplification of the DNA fragment delineated by the two primers. The synthesis of DNA during extension steps is driven by the thermostable DNA polymerase from *Thermus aquaticus* (*Taq* polymerase). By choosing sequences that are conserved among widely divergent species, it is possible to design "universal" primers. Such primers facilitate evolutionary analysis because they can amplify a particular DNA fragment in virtually all members of a major taxonomic group (Kocher *et al.* 1989; Kocher and White 1989).

PCR can be used to amplify DNA from minute amounts of starting template, including single hairs (Vigilant *et al.* 1989). Thus, the application of universal primers has important implications for PCR hygiene. Given the sensitivity of PCR and its ability to synthesise millions of DNA fragments, contamination from both exogenous sources and previous amplifications is a constant danger. Furthermore, studies have shown that contamination may be cryptic, especially when examining distantly related taxa (Derr *et al.* 1992).

Rigorous procedures are required to prevent PCR contamination. In this study, the following precautions were observed in the extraction and amplification of DNA. It is essential to physically separate PCR preparation from the analysis of PCR products (Erlich 1989). Glassware for PCR procedures should be heat-treated (200° C, 4 h) to destroy DNA. Where possible, plasticware and solutions should be autoclaved; all PCR reagents must be stored as sub-aliquots (Kwok and Higuchi 1989; Cimino *et al.* 1990). Non-sterile solutions are treated with short-wave ultraviolet radiation for 5-20 min to damage any DNA that may be present (Sarkar and Sommer 1990). To prevent sample carry over, positive displacement pipettes or plugged pipette tips must be used when transferring DNA solutions. To avoid human contamination of solutions, disposable gloves, dust-masks and hair-nets should be worn at all times and changed frequently. Laboratory surfaces are sterilised with 1 M HCl or 10% bleach solution prior to sample preparation. A negative control or "blank" containing no template DNA is included in all DNA extraction and PCR experiments.

A final means of avoiding contamination involves the design of PCR primers. In experiments where the template DNA/primer complementarity is strong, contamination is generally not a serious problem. However, with distantly related taxa, template/primer mismatches may occur. In such cases, any contaminating DNA with strong complementarity will be preferentially amplified. This problem may be circumvented by designing primers with a high degree of identity to the sequences of the target taxa (Derr *et al.* 1992; Finnerty and Block 1994a).

2.2.2 Collection and preservation of specimens

PCR can be used to amplify DNA extracted from ancient dried animal specimens that contain minute amounts of template DNA (Ellegren 1991). In addition, protocols have been developed to extract DNA from formalin-fixed and paraffin-embedded fish tissues (Shiowaza *et al.* 1992). However, such preservation techniques invariably degrade DNA. Ideally, DNA should be extracted from fresh or frozen tissue. Alternatively, for studies of widely dispersed taxa, ethanol is generally favoured as a preservative as it eliminates the necessity of carrying dry ice or liquid nitrogen into the field.

Where possible, DNA was extracted from fresh muscle or liver tissue. However, in most cases, specimens were stored frozen (liquid nitrogen or -80° C freezer) or preserved in ethyl alcohol (70-95%) prior to DNA extraction. To reduce potential sources of contamination when removing liver and muscle tissue, care was taken to avoid the skin and alimentary tract of specimens. Specimens were frozen whole in aluminium foil, or tissue samples were frozen in cryotubes. Ethanol preserved specimens were stored in sterilised glass containers. The tissue types and preservation details of samples used for DNA sequencing are shown in Table 2.1.

2.2.3 DNA extractions

DNA was extracted from 27 galaxioid species (Table 2.1). Four extraction techniques were used to isolate and purify DNA from galaxioid fish tissue. The first two techniques are based on the phenol-chloroform method. Phenol cleans aqueous DNA by denaturing proteins which subsequently flocculate and appear as a milky solid; chloroform is used to remove residual phenol from the extraction. The latter two extraction techniques use a matrix to purify total DNA.

Phenol chloroform with mitochondrial enhancement

DNA was extracted from fresh, frozen or alcoholic tissue using a modification of the protocol described in Palumbi *et al.* (1991). This method is ideal for studies of mitochondrial DNA as much of the nuclear DNA is eliminated early in the procedure.

Table 2.1 Details of tissue type and preservation history of samples used for DNA sequencing.

Species	Origin	Locality	Latitude	Longitude	Collector	Tissue	Preservation
Retropinna tasmanica	Tasmania	Derwent R.	42°45'S	147°13'W	P. Boxall	Liver	Frozen
Lepidogalaxias salamandroides	Western Australia	Chesapeke Rd.	34°38S	116°06W	H. Gill	Muscle	Ethanol
Lovettia sealii	Tasmania	Northern Tas.	41°S	147°W	J. Purser	Muscle	Ethanol
Aplochiton zebra	East Falkland	Deep Arroja R.	51°58'S	59°12'W	J. Smith	Muscle	Ethanol
Galaxias argenteus	New Zealand	unknown	unknown	unknown	T. Eldon	Muscle	Ethanol
Galaxias auratus	Tasmania	L. Crescent	42°08'S	147°09'W	B. Mawbey	Liver	Fresh
Galaxias brevipinnis	Tasmania	Snug R.	43°04'S	147°12'W	J. Waters	Liver	Frozen
Galaxias cleaveri	Tasmania	Allens Ck.	43°03'S	147°53'W	D. Crook	Muscle	Frozen
Galaxias fasciatus	New Zealand	unknown	unknown	unknown	T. Eldon	Muscle	Ethanol
Galaxias fontanus	Tasmania	Swan R.	41°50'S	148°06'W	J. Waters	Liver	Fresh
Galaxias johnstoni	Tasmania	Clarence Lagoon	42°04'S	146°18'W	R.W.G. White	Liver	Frozen
Galaxias maculatus	Tasmania	Sandy Bay Riv.	42°53'S	147°20'W	J. Waters	Liver	Fresh
Galaxias maculatus	Saunders Island	Grey Duck Pond	51°20'S	60°10'E	R. Maddocks	Liver	Ethanol
Galaxias maculatus	West Falkland	Shallow Harbour	51°44'S	60°32'E	M. Marsh	Livwe	Ethanol
Galaxias olidus	Victoria	Glenelg R.	37°10'S	141°50'W	A. Sanger	Liver	Frozen
Galaxias parvus	Tasmania	48 Ck.	42°58'S	146°21'W	R. W. G. White	Liver	Frozen
Galaxias paucispondylus	New Zealand	Wilberforce R.	43°02'S	171°10'W	T. Eldon	Muscle	Ethanol
Galaxias truttaceus	Tasmania	Allens Ck.	43°03'S	147°53'W	J. Waters	Liver	Frozen
Galaxias vulgaris	New Zealand	South Island	unknown	unknown	C. P. Mitchell	Muscle	Ethanol
Galaxias zebratus	South Africa	Noetzie R.	34°04'S	23°11'E	P. Skelton	Muscle	Ethanol
Galaxias zebratus	South Africa	Olifants R.	33°S	19°E	D. Impson	Liver	Ethanol
Galaxias zebratus	South Africa	Eerste R.	34°S	18°E	J. Cambray	Liver	Ethanol
Galaxias zebratus	South Africa	Kouga R.	33°53'S	23°51'E	J. Cambray	Liver	Ethanol
Galaxias zebratus	South Africa	Krom R.	33°52'S	23°57'E	J. Cambray	Liver	Ethanol
Neochanna apoda	New Zealand	Hamilton	37°48'S	174°52'W	B. Hicks	Muscle	Ethanol
Neochanna burrowsius	New Zealand	Canterbury	44°S	172°W	T. Eldon	Muscle	Ethanol
Paragalaxias mesotes	Tasmania	Arthurs L.	42°01'S	146°56'W	J. Ovenden	Liver	Frozen
Paragalaxias julianus	Tasmania	Carter's L.	41°52'S	146°31'W	J. Ovenden	Liver	Frozen
Galaxiella munda	Western Australia	Pemberton	34°26'S	116°03'W	R.W.G. White	Muscle	Frozen
Galaxiella nigrostriata	Western Australia	Chesapeke Rd.	34°38S	116°06W	H. Gill	Muscle	Ethanol
Galaxiella pusilla	Tasmania	Forester Lodge	41°S	148°W	P. Humphries	Muscle	Ethanol
Brachygalaxias bullocki "gothei"	Chile	Talca	36°S	72°E	K. Busse	Muscle	Ethanol
Nesogalaxias neocaledonicus	New Caledonia	L. en Huit	22°S	166°W	C. Pöllabauer	Muscle	Ethanol

The tissue sample (0.1-0.2 g) was rinsed briefly in a microcentrifuge tube containing 1 ml of sterile distilled water (SDW). The water was drained and the tissue was macerated in 300 µl of buffer (0.1 M Tris, 0.15 M NaCl, pH 7.5) with a sterile micropestle. The homogenate was centrifuged at 6,000 rpm to pellet nuclei and cell debris. The supernatant was transferred to a 1.5 ml microcentrifuge tube containing 300 µl of lysis buffer (0.2 M NaCl, 0.02 M Tris, 1 mM EDTA, 2% SDS) and inverted for one min. The sample was centrifuged at 13,000 rpm for 5 min. The supernatant, containing mtDNA, was mixed with 500 µl of equilibrated phenol and centrifuged at 13,000 rpm for 10 min. The aqueous phase was transferred to a new microcentrifuge tube. The extraction was repeated, initially with 500 µl of 25:24:1 phenol/chloroform/isoamyl alcohol (IAA) and finally with an equal volume of 24:1 chloroform/IAA. The extracted sample was subsequently purified using one of two techniques.

The first technique involved ethanol precipitation. The supernatant was combined with a 0.5 volume of 7.5 M ammonium acetate and 2 volumes of absolute ethanol. This solution was incubated at room temperature for 10 min to precipitate the DNA which was pelleted at 13,000 rpm for 10 min. The supernatant was discarded and the DNA pellet was soaked in 70% ethanol for 10 min before a final 10 min spin at 13,000 rpm. The ethanol was discarded and the pellet was vacuum dried with punctured parafilm for 10 min. The pellet of purified DNA was resuspended in 100 µl SDW and stored at -20° C.

Alternatively, the aqueous phase from the phenol-chloroform extracted sample was purified and concentrated by filtration. The sample was transferred to a *Millipore Ultrafree-MC* filter microcentrifuge tube and centrifuged at 7,000 rpm for 15 min. The DNA was then washed: 500 µl of SDW was added to the tube which was centrifuged at 7,000 rpm for 20 min. This step was repeated twice and the final spin was continued until the sample volume was reduced to 100 µl. This purified DNA sample was stored at -20° C.

CTAB extractions

For some ethanol preserved specimens, DNA was extracted from muscle tissue homogenised in a hexadecyltrimethylammonium bromide (CTAB) buffer. This protocol, adapted from Saghai-Maroof *et al.* (1984), is designed for animal tissues where there is a relatively large amount of protein and relatively little DNA. CTAB forms a complex with DNA at low salt concentrations; this increases the ease with which enzyme inhibiting contaminants are removed from the supernatant (Reichardt and Rogers 1994).

Approximately 0.1 g of muscle tissue was rinsed in SDW and placed in 600 µl of CTAB buffer (0.1 M Tris-HCl pH 8, 0.02 M EDTA, 1.4 M NaCl, 2% CTAB, 0.2% 2-mercaptoethanol). The sample was homogenised with a sterile micropestle and 5 µl of 20 $mg.ml^{-1}$ proteinase K was added. The homogenate was incubated at 65° C for two h with occasional vortex mixing. The homogenate was then extracted with 600 µl of 25:24:1 phenol/chloroform/IAA and centrifuged at 13,000 rpm for 10 min. The aqueous supernatant was removed and the extraction was repeated. The sample was extracted for a final time with an equal volume of 24:1 chloroform/IAA and centrifuged at 13,000 rpm for 30 sec. The supernatant was removed and combined with 1.5 volumes of cold (-20° C) isopropanol in a new microcentrifuge tube. The sample was incubated at -20° C for at least 12 h. The precipitated DNA was pelleted by centrifugation at 13,000 rpm for 20 min at 4° C. The supernatant was discarded and replaced with 500 µl of cold (-20° C) 70% ethanol. After gentle mixing, the sample was centrifuged at 13,000 rpm for 10 min. The supernatant was removed and the pellet was dried *in vacuo* for 30 min. The DNA pellet was resuspended in 50 µl of SDW and stored at -20° C.

Chelex extractions

The chelex extraction technique was originally developed for forensic PCR applications (Walsh *et al.* 1991). It can be used to isolate DNA from very small amounts of starting tissue. In this protocol, a boiling step lyses cells and denatures proteins. Cell lysis products that are likely to inhibit PCR amplification are absorbed by a chelating resin.

Approximately 2 mm^3 of tissue was rinsed in a microcentrifuge tube containing 1 ml of SDW. The sample was transferred to a clean sterile surface and minced with a sterile scalpel blade. The macerated tissue was transferred to a 1.5 ml microcentrifuge tube containing 200 µl of resuspended 6% *Chelex 100* solution (*Instagene Purification Matrix, Biorad*); 1-2 µl of 20 $mg.ml^{-1}$ proteinase K was added. The sample was incubated at 56° C for 2-24 h with occasional flick mixing. After incubation, the tube was vortexed for 10 sec, placed in a boiling waterbath for 8 min and again vortexed for 10 sec. Finally, the tube was centrifuged at 13,000 rpm for 2 min to pellet the resin and bound impurities. The supernatant, containing the DNA, was removed and stored at -20° C.

Bresa-Clean extractions

A final total DNA extraction technique used the commercially available *Bresa-Clean* kit (*Bresatec*). This method uses a silica matrix (*Bresabind*) to bind DNA in a chaotropic

solution (*Bresasalt*). Proteins in solution are denatured and do not bind to the matrix, so phenol extraction and ethanol precipitation are not necessary.

Rinsed alcoholic tissue was minced with a scalpel blade, placed in 250 µl of digestion buffer (0.1 M NaCl, 0.05 M Tris, 0.01 M EDTA, 0.0001 g proteinase K, 1% SDS) and incubated at 37° C for 24 h. The digested tissue was centrifuged for 5 min at 13,000 rpm and the supernatant was added to 750 µl of *Bresasalt*. *Bresabind* matrix was resuspended by vortexing for 1 min and 5 µl was mixed with the sample. After a 10 min incubation at room temperature with regular mixing, the sample was centrifuged at 13,000 rpm for 1 sec. the supernatant was replaced with 750 µl of cold (4° C) *Bresawash* solution and the pellet (matrix plus DNA) was resuspended by vortexing. The *Bresawash* step was repeated and the pellet was subsequently vacuum dried for 10 min. The DNA was eluted by resuspending the pellet in 50 µl of SDW, incubation at 50° C for 10 min and centrifugation at 13,000 rpm for 1 min. The supernatant was carefully drawn off and placed in a 0.5 ml microcentrifuge tube. The elution step was repeated to give a final extracted volume of 100 µl which was stored at -20° C.

2.2.4 PCR primers

Synthetic oligonucleotide primers were used to amplify partial sequences of two mitochondrial genes. For most specimens, a 307 base pair (bp) section of the mitochondrial cytochrome b was amplified with the primers of Kocher *et al.* (1989): L14841 (5' CCATCCAACATCTCAGCATGATGAAA 3') and H15149 (5' CCCTCAGAATGATATTTGTCCTCA 3'). However, this segment failed to amplify in several species due to mismatches with the L14841 primer. To rectify this, a new light strand primer L14848 (5' ATATCTCAGTCTGGTGAAACTTTGG 3') was designed based on the sequence of *Salmo fibreni* (Patarnello *et al.* 1994). This primer was used with H15149 to amplify a 301 bp fragment. A few specimens still failed to amplify with this new primer. Finally, a third light strand primer L14724 (5' CGAAGCTTGATGAAAAACCATCGTTG 3', Meyer *et al.* 1990) was used with H15149 to successfully amplify a larger 425 bp fragment.

In addition, an approximately 600 bp fragment of the mitochondrial 16S rRNA gene was amplified from all specimens using the universal primers 16Sar (5' CGCCTGTTTATCAAAAACAT 3') and 16Sbr (5' CCGGTCTGAACTCAGATCACGT 3') from Palumbi *et al.* (1991). An internal H-strand primer was designed for PCR and sequencing: JW3 (5' GCGCTGTTATCCCTAGGGTA 3').

2.2.5 PCR amplification

Double-stranded amplifications were performed in 500 μl microcentrifuge tubes with 50 μl volumes. Reactions contained 5 μl of 10x buffer (500 mM KCl, 100 mM Tris-HCl pH 9, 10% Triton X-100), 1-3 mM $MgCl_2$, 800 μM of dNTPs (200 μM of each), 0.5 μM of each primer, and 0.5-1.5 units of *Amplitaq* DNA polymerase (*Perkin-Elmer Cetus*). Before the addition of primers, *Taq* polymerase and template DNA, the PCR cocktail and reaction tubes were exposed to shortwave UV radiation for 5-10 min to help eliminate any contaminating DNA. Reactions were overlain with two drops of UV-treated paraffin oil to prevent condensation. After centrifugation at 13,000 rpm for 1 sec, 2 μl of purified DNA template was added through the oil layer and tubes were flick mixed.

The concentration of $MgCl_2$ in the reaction buffer is a variable that can greatly influence amplification success. Optimal concentrations vary for different target sequence and primer combinations (Saiki 1989). To optimise PCR conditions for new templates and primers, a titration series $MgCl_2$ concentrations was prepared (Table 2.2). A 25 mM $MgCl_2$ stock was thoroughly thawed and vortexed prior to use as storage at -20° C results in stratification and precipitation.

Table 2.2 Table showing the set up of a typical PCR experiment with 50 μl reaction volumes A positive control is included to monitor the quality of the PCR reagents while a negative control and negative extraction test for contamination of the reactions. The PCR cocktail contains *10x buffer*, dNTPs, primers and diluted *Taq* DNA polymerase. In this experiment, tubes 1-5 represent a dilution series to determine the optimal $MgCl_2$ concentration.

Tube	Sample	[$MgCl_2$]	25 mM $MgCl_2$	SDW	PCR cocktail	Extract
1	*G. parvus*	1 0 mM	2.0 μl	6 0 μl	40.0 μl	2 0 μl
2	*G. parvus*	1.5 mM	3.0 μl	5.0 μl	40.0 μl	2.0 μl
3	*G. parvus*	2.0 mM	4.0 μl	4.0 μl	40 0 μl	2.0 μl
4	*G. parvus*	2.5 mM	5.0 μl	3.0 μl	40.0 μl	2 0 μl
5	*G. parvus*	3 0 mM	6.0 μl	2.0 μl	40.0 μl	2.0 μl
6	-ve extract	2.0 mM	4.0 μl	4.0 μl	40 0 μl	2.0 μl
7	+ve control	2 0 mM	4.0 μl	4 0 μl	40 0 μl	2 0 μl
8	-ve control	2.0 mM	4.0 μl	6 0 μl	40.0 μl	0.0 μl

Amplifications were carried out in a *Corbett Research FTS-320* thermal sequencer. A variety of cycling parameters were used; stringency was modified to improve the quality and yield of amplified products. Amplifications with 16S rRNA primers involved 40 cycles of denaturation at 94° C (30 sec), annealing at 50-55° C (1 min) and extension at 72° C (2 min). The same parameters were used to amplify cytochrome b fragments for several species. However, for some species, amplifications were strengthened by lowering the annealing temperature to 45° C. Alternatively, primer annealing was increased by lowering the annealing temperature to 45° C and extension temperature to 68° C for the first 10 cycles, followed by 30 cycles at standard

temperatures (55° C and 72° C). In all cases, the initial denaturation step and the final extension step were lengthened to 5 min and 10 min respectively.

To improve PCR yield and specificity, some templates were amplified with the Hot Start technique. This method reduces the primer oligomerisation and mis-priming that occur when reactants are mixed before amplification (D'Aquila *et al.* 1991). A PCR mix containing $MgCl_2$, dNTPs, primers and 10x buffer was added to a 500 µl microcentrifuge tube. An *AmpliWax PCR Gem* (*Perkin Elmer*) was dispensed into the tube and melted by incubation in an 80° C waterbath for 5 min. The tube was then incubated at room temperature (2 min) and a PCR mix containing 10x buffer, SDW, *Taq* polymerase and DNA template was added on top of the now solidified wax. Thermal cycling melted the wax and mixed the separated PCR components at 94° C. The *AmpliWax PCR Gem* removed the requirement for mineral oil.

Amplified products were displayed on an agarose gel and examined for specificity and yield. The molecular weight of PCR products was checked against an electrophoresis size standard: 1 µl of SPP-1 phage DNA (*Bresatec*) digested with *Eco RI*. A 1 µl aliquot of loading buffer (bromophenol blue and xylene cyanole) was mixed with 2 µl of each completed reaction. Electrophoresis of samples was performed for 25 min at 100 V through a 1% agarose minigel in TAE buffer (0.04 M Tris, 0.019 M glacial acetic acid and 1 mM EDTA pH 7.8). The minigel was stained with 0.4 $\mu g.ml^{-1}$ ethidium bromide for 20 min and photographed on a 300 nm transilluminator with *Polaroid Type 667* film (*ISO 3000*).

2.2.6 Purification of PCR products

Before sequencing, double stranded PCR amplifications must be purified. In this study, three commercial purification kits were used. All these methods use a matrix to bind DNA during purification. One of the purification kits (*Wizard, Promega*) can directly purify DNA from PCR reactions while the other two techniques, *Geneclean II* (*BIO 101*) and *Bresa-clean* (*Bresatec*), require PCR products to be initially purified by agarose gel electrophoresis. The remaining 48 µl of the successful PCR reaction was run through a 1.4% agarose/TAE minigel (pH 7.5) for 1 hour at 100 V. The minigel was stained for 20 min in 150 ml of TAE containing 0.4 $\mu g.ml^{-1}$ ethidium bromide. The stained band of PCR product was excised under long wave UV light and placed in a preweighed microcentrifuge tube.

Geneclean

The excised agarose slice was melted at 55° C in 3 volumes (w:v) of sodium iodide solution. The *Glassmilk* matrix was resuspended by vortexing for 1 min and 5 µl was

added to the DNA-NaI solution. This mixture was incubated on wet ice for 10 min with regular mixing. The suspension was centrifuged at 13,000 rpm for a few sec to pellet the matrix (and bound DNA) and the supernatant was removed. The pellet was thoroughly resuspended in 500 µl of cold (-20° C) *New Wash* solution by repeat pipetting. The suspension was centrifuged at 13,000 rpm for a few sec and the supernatant was removed. The matrix was washed with *New Wash* twice more and finally centrifuged for 1-2 min. The supernatant was removed with care taken to avoid the pelleted matrix. The microcentrifuge tube was covered with punctured parafilm and the pellet was vacuum dried for 5 min. Two elutions were performed; each involved resuspension in 10 µl SDW, 5 min incubation at 50° C and a 30-60 sec spin at 13,000 rpm. The supernatant was removed with a pipettor, avoiding the pelleted *Glassmilk*. The two eluants were combined to give 20 µl of purified DNA which was stored at 4° C.

Bresa-Clean

The excised agarose slice was melted at 55° C in 3 volumes (w:v) of *Bresasalt* solution. The *Bresa-bind* matrix was resuspended by vortexing for 1 min and 5 µl was added to the DNA-*Bresasalt* solution. This mixture was incubated at room temperature for 5 min with regular inversion mixing. The suspension was centrifuged at 13,000 rpm for a few sec to pellet the matrix (and bound DNA) and the supernatant was removed. The pellet was thoroughly resuspended in 500 µl of cold (4° C) *Bresa-Wash* solution by repeat pipetting. The suspension was centrifuged at 13,000 rpm for a few sec and the supernatant was removed. The matrix was washed with *Bresa-Wash* twice more and finally centrifuged for 1-2 min. The supernatant was carefully removed, avoiding the pelleted matrix. The microcentrifuge tube was covered with punctured parafilm and the pellet was vacuum dried for 5 min. Two elutions were performed; each involved resuspension in 10 µl SDW, 5 min incubation at 50° C and a 30-60 sec spin at 13,000 rpm. The supernatants of each elution were carefully removed and combined to give 20 µl of purified DNA which was stored at 4° C.

Wizard

This technique uses a *Wizard Minicolumn* (*Promega*) to directly purify products from PCR reactions. A resin binds DNA while impurities such as primer oligomers are filtered out of solution by centrifugation.

The aqueous layer from a successful PCR reaction (48 µl) was mixed with 100 µl of *Direct Purification Buffer* in a 1.5 ml microcentrifuge tube. A 1 ml aliquot of resuspended *Resin* was added and the resulting suspension was vortexed briefly three times over a one min period. The suspension was transferred to the barrel of a 2 ml

disposable syringe and pushed into an attached *Minicolumn* by gently inserting the plunger. The *Resin*/bound DNA was washed: 2 ml of 80% isopropanol was added to the syringe barrel and pushed through the *Minicolumn*. The *Minicolumn* was placed in a 1.5 ml microcentrifuge tube and centrifuged at 13,000 rpm for 20 sec to dry the *Resin*. To elute the DNA from the Resin, 25 µl of SDW was added to the *Minicolumn* which was incubated at room temperature for 5 min. Finally, the *Minicolumn* was placed in a microcentrifuge tube and centrifuged for 20 sec at 13,000 rpm to remove the purified DNA which was stored at 4° C.

2.2.7 DNA sequencing

Manual sequencing

Most purified PCR products were sequenced using one of two techniques, both based on the enzymatic Dideoxy method developed by Sanger *et al.* (1977). One of these methods involves direct incorporation of radioactive nucleotides while the other uses radioactive endlabelling. The Dideoxy technique uses a DNA polymerase to synthesise a complimentary copy of a single stranded template. DNA synthesis is initiated at the 3' end of an oligonucleotide primer annealed to the template DNA. Deoxynucleotides (dNTPs) are added to the growing chain in a 5'-3' direction. A new dNTP is incorporated when its 5'-phosphate group forms a phosphodiester bond with the 3'-hydroxyl group of the previous dNTP. However, when a dideoxynucleotide (ddNTP) is incorporated, chain elongation is terminated. This is because the ddNTP lacks the 3'-hydroxyl group required to bond with subsequent nucleotides.

Sequence determinations are carried out as a set of four separate reactions, each of which contains all four dNTPs but only one ddNTP. As a result, chain synthesis is terminated selectively at guanine (G), adenine (A), thymine (T) or cytosine (C), corresponding to the ddNTP present. The various nucleotide chains that are formed have a common origin, but end in different nucleotides. The average length of sequencing products can be altered by changing the relative proportions of dNTPs and ddNTPs as this increases or decreases the likelihood of incorporating a ddNTP. Sequencing products are separated according to size by gel electrophoresis and visualised by autoradiography.

Sequenase Version 2.0

Most purified PCR products were sequenced using a modification of the protocol recommended for use with *Sequenase Version 2.0* (*United States Biochemical*). This technique uses a modified T7 DNA polymerase (*Sequenase*) to directly incorporate radioactive nucleotides during two separate chain extension steps. Initially, template DNA is denatured and annealed to the appropriate primer. The first step (labelling)

involves extension of the annealed primer using limiting concentrations of the dNTPs, including radiolabelled dATP. The second step (termination) involves further chain extension until the incorporation of a ddNTP ends DNA synthesis. The detergent Non-idet P-40 was included in the reaction mixes as this has been shown to improve sequence quality (Bachmann *et al.* 1990).

Sequencing reactions were carried out in 500 µl microcentrifuge tubes. Two microlitres of *5x Reaction Buffer*, 1 µl of 20% Non-idet P-40 and 1 µl of 10 µM primer solution was combined with 7 µl of purified double-stranded DNA template. This 11 µl volume was placed in a boiling waterbath for 3 min and then snap-frozen in liquid nitrogen or a dry ice/ethanol (-20° C) bath. The tube was flash-spun (13,000 rpm for 1 sec) to collect condensation and stored on wet ice. For the first labelling step, a mixture comprising 1 µl of 0.1M DTT, 2 µl of Labelling Mix (diluted 1:6 with SDW), 0.5 µl of $\propto^{35}$S-dATP (12.5 $\mu Ci.\mu l^{-1}$) and 2 µl of *Sequenase* (diluted 1:8 with *Dilution Buffer*) was added to the annealed reaction. This labelling reaction was flick mixed and incubated at room temperature (<20° C) for 2-5 min. Subsequently, 3.5 µl of the completed labelling reaction was added to each of 4 prewarmed (37° C) microcentrifuge tubes containing 2.5 µl of one of the four termination mixes (ddGTP, ddATP, ddTTP or ddCTP). After a 5 min incubation at 37° C, reactions were terminated by the addition of 4 µl of *Stop Solution* (containing EDTA and formamide). The completed sequencing reactions were centrifuged at 13,000 rpm for 1 sec and stored at -20° C prior to electrophoresis and autoradiography.

FMOL DNA Sequencing System

Some PCR products were cycle sequenced with the *fmol DNA Sequencing System* (*Promega*). Cycle sequencing is an alternative enzymatic sequencing technique which uses the thermostable DNA polymerase isolated from *Thermus aquaticus* (Taq DNA polymerase). Similar to the PCR, this technique uses a thermocycling apparatus for annealing, extension and denaturation steps. Cycle sequencing has several advantages: relatively little starting template is needed, high temperatures reduce the potential for secondary structure in sequencing products, and annealing stringency can be modified to suit the sequencing primer. The *fmol* system uses radioactive end-labelling of primers to detect sequencing products. In contrast to direct incorporation used in the *Sequenase* protocol, endlabelling generates data very close to the sequencing primer.

Endlabelling and cycle sequencing reactions were performed in 500 µl microcentrifuge tubes. Radioactive endlabelling reactions comprised 3 µl of $\propto^{33}$P-dATP (10 $\mu Ci.\mu l^{-1}$), 1 µl of *T4 Polynucleotide Kinase* (PnK, 5-10 $U.\mu l^{-1}$), 1 µl of *PnK 10x buffer* (*New England Biolabs*), 2 µl of SDW and 3 µl of 10 µM primer. The reaction was incubated

at 37° C for 30 min and then at 90° C for 2 min to inactivate the PnK. The microcentrifuge tube was centrifuged at 13,000 rpm for 1 second to collect condensation and stored at -20° C. Cycle sequencing reactions comprised 5 µl of DNA template, 1.5-2.5 µl of endlabelled primer, 5 µl of *fmol 5x Sequencing Buffer*, 1 µl of Sequencing Grade *Taq* DNA Polymerase (5 $U.\mu l^{-1}$) and SDW in a 17 µl volume. Four microlitres of this cocktail was added to each of 4 tubes containing the appropriate d/ddNTP mix. Reactions were overlain with 1 drop of mineral oil and placed in a preheated (94° C) thermal cycler. The subsequent thermal cycling parameters consisted of denaturation (94° C, 30 sec), annealing (50° C, 30 sec) and extension (72° C, 60 sec). At the completion of 30 cycles, 3 µl of *fmol Stop Solution* was added to each tube to terminate the reactions. Sequencing products were stored at -20° C prior to electrophoresis and autoradiography.

Automated sequencing

Towards the end of this study, some purified PCR reactions were sequenced with an automated technique. This method involves a single tube cycle sequencing reaction with four dye-labelled terminators and determines sequences without autoradiography. Initially, the concentration of purified DNA was quantified with a DNA fluorometer (*Hoefer*). Sequencing reactions were constructed as recommended using the ABI Prism Dye-Terminator kit (*Perkin-Elmer*). Reactions contained 8 µl of dye-terminator ready reaction mix, 3.2 pmol of primer and 50 ng of amplified DNA template, in a total volume of 20 µl. Thermal cycling performed in a GeneAmp PCR Systems 9600 cycler (*Perkin-Elmer*) with 30 cycles of denaturation (96° C, 19 sec), annealing (50° C, 5 sec) and extension (60° C, 4 min). Completed sequencing reactions were purified by ethanol precipitation and run on an ABI Prism 377 autosequencer (*Perkin-Elmer*). In this method, samples were loaded into a single lane on a polyacrylamide gel. A laser scanned across the gel and detected the fluorescent dye-labelled fragments. For each sample, sequencing reactions were performed from both primers to allow complementary strands to be read.

2.2.8 Electrophoresis and autoradiography

Polyacrylamide electrophoresis of radioactively-labelled sequencing products was performed with a *Sequi-Gen Nucleic Acid Sequencing Cell* (*BIO-RAD*). This apparatus includes two 50 cm glass plates, of which one was coated with *Gel Slick* (*AT Biochem*) and the other coated with *Bind Silane*. The glass plates were separated by wedge-shaped spacers (0.25 mm thick at the top, thicker at the bottom). Gels were run in TBE electrophoresis buffer (89 mM Tris, 89 mM boric acid, 25 mM EDTA pH 8.2). Approximately 40 ml of degassed 6% acrylamide/TBE was mixed with 40 µl of 25% APS and 40 µl of TEMED and poured between the glass plates. Polymerisation

occurred in approximately 15 min, but gels were aged for at least 3 h prior to running. A 48 tooth shark-tooth comb was inserted into the top of the gel to create wells for sample loading. Approximately 2 μl of loading dye (bromophenol blue, xylene cyanole) was loaded to check the quality of the wells. The gel was pre-electrophoresed at 60 W for one hour or until a gel temperature of 50° C was attained.

Prior to sample loading, wells were flushed out with TBE buffer to remove urea and unpolymerised acrylamide. Sequenced DNA samples were denatured at 90° C for 2 min and 2.5 μl of each reaction was loaded onto the gel in the order GATC. Gels were run at 60 W for 15 min and then at 55 W for between 1.5 and 4 h, depending on the size of the sequencing products to be separated. At the completion of the run, the gel plates were separated and the gel was soaked in 10% glacial acetic acid for 30-60 min with frequent agitation to remove urea. Gels were dried for 2 h in a 70° C oven and subsequently exposed to X-ray film in a light-proof box. Exposure times varied from as short as 18 h for α^{33}P-dATP-labelled products to as long as 1 week for α^{35}S-dATP-labelled products. Autoradiographs were read over a light box and light strand sequences were recorded in a 5'-3' direction. Loading in the order GATC enabled complimentary sequences to be read by inverting the autoradiograph.

2.2.9 Sequence alignment and phylogenetic analysis

To increase confidence that data obtained were galaxioid mtDNA rather than exogenous (e.g. human DNA) or endogenous (parasite DNA) contamination, sequences were compared with those in molecular databases. The Australian National Genomic Information Service (ANGIS) was accessed via Telnet. The BLAST match option was used to retrieve nucleotide sequences in the EMBL and Genebank databases possessing the highest degree of similarity to the putative galaxioid sequences.

Galaxioid mtDNA sequence alignments were performed with the alignment program CLUSTAL (Higgins and Sharp 1988) implemented in Sequence Navigator (Applied Biosystems, Perkin Elmer). This program initially uses an alignment algorithm to calculate pairwise sequence similarity. Sequences are then progressively aligned in order of similarity by inserting gaps. The relative weight of gaps and mismatches is specified by the user. For phylogenetic analysis, mismatches are generally favoured over gaps to avoid unrealsitic alignments (e.g. one sequence having gaps at each position where another has nucleotides). A variety of gap/mismatch weighting schemes were implemented, and the resulting alignments were compared. Final alignments were generated with a penalties of ten for opening a gap, and five for extending a gap. Completed alignments were visually inspected for misalignments between closely related taxa, and some minor adjustments were made. To minimise the impact of

particular alignments on phylogenetic conclusions, alignment gaps were subsequently treated as missing data.

Galaxioid 16S rRNA sequences were compared with large subunit (LSU) rRNA primary sequences and secondary structures from an electronic database (http://pundit.colorado.edu:8080/root.html). This database presently contains secondary structures for the mitochondrial LSU rRNA of two animals, of which only one is a chordate (*Bos taurus*; Gutell *et al.* 1993). In an attempt to estimate the distribution of paired (stem) and unpaired (loop) regions in galaxioid 16S rRNA, sequences were manually folded around the cow 16S rRNA secondary structure.

Genetic variation was examined both within and between taxa. The computational requirements of phylogenetic analysis increase exponentially with increasing numbers of nucleotide sequences, irrespective of the method used to recover phylogeny. For this reason, it was decided to compartmentalise the analysis of galaxioid mtDNA sequences into three sections. These were (1) intraspecific phylogeography; (2) phylogeny of the Galaxiinae; (3) phylogeny of the Galaxioidei. For species in which a high level of genetic diversity was detected, the two most divergent sequences were included as representatives of that of taxon in the phylogenetic analysis of the Galaxiinae. This strategy has the advantage of helping to reduce long branch effects while limiting the number of sequences in the analysis.

Maximum likelihood searches were conducted with "random addition" and used "empirical base frequencies". Optimal transition (TI) to transversion (TV) biases were determined with maximum likelihood analysis. Because of the high computational demands of this method, representative subsets of nine sequences from each data set were analysed under a range of expected TI/TV biases. For each data set, the TI/TV bias producing the tree with the greatest likelihood was used in subsequent phylogenetic analyses.

In distance analyses, genetic divergence between paired sequences was calculated with the maximum likelihood algorithm of Felsenstein (1993) which is implemented in the program DNADIST (PHYLIP). The "empirical base frequencies" option and a single category of substitution rates were used. The neighbor-joining method (Saitou and Nei 1987) in PHYLIP was used to construct phylogenetic trees from distance matrices. Confidence in tree topology was assessed by bootstrapping with the SEQBOOT and CONSENSE programs.

Nucleotide sequences that are highly conserved due to functional constraints may be saturated with variation at the few sites free to vary. In order to discriminate between true phylogenetic signal and random noise from saturated sequences, random-treelength distribution was examined prior to parsimony analysis. The $g1$ statistic, as implemented in PAUP version 3.1 (Swofford 1993), was used to measure the skewness of the distribution. The statistical significance of $g1$ values was determined through comparison with critical values listed in Hillis and Huelsenbeck (1992).

Parsimony analyses were performed with the program PAUP. For analyses involving fewer than a dozen taxa or sequences, either the "exhaustive" search option or "branch and bound" option was selected. These methods are guaranteed to find the most parsimonious (mp) tree(s). In contrast, large data sets were analysed with the "heuristic" search option. To increase the chance of this method recovering the shortest tree(s), searches were repeated 30 times with random addition of taxa. When more than one mp tree was recovered, a strict-consensus was also computed.

Confidence in mp tree topology was assessed with one of two techniques. Firstly, the bootstrapping option was selected, with heuristic searches on 500 replicate data sets. Secondly, Bremer support values were determined by examining near most parsimonious (nmp) trees and by enforcing topological constraints. Initially, the "keep all trees ≤ length" was used to recover all trees within six steps of the mp tree. The filtering option was then used to retain all trees within one, two, three, four, five and six steps of the mp tree. A strict consensus was generated for each filtered set of trees. In this way, the number of extra steps required to disrupt each clade was determined. Alternatively, Bremer support values of particularly well supported clades (> six steps) were determined by enforcing topological constraints. Specifically, heuristic searches were conducted with the "converse constraints" option to find the shortest tree in which a particular clade was not supported.

For parsimony analyses, several alternative weighting schemes were trialed. Differential weighting of codon positions was performed with the "set character weights" option. The optimal TI/TV bias determined from maximum likelihood analysis was implemented in parsimony analyses with the "stepmatrix" option in PAUP. Alternatively, The expected/observed ratio (EOR) weighting strategy of Knight and Mindell (1993) was used to preferentially weight rare classes of substitutions. To determine EOR weights, the observed numbers of each substitution was determined from pairwise comparisons of a subset of five sequences. The expected occurrence of each class of substitution was determined by multiplying the total number of transitions

or transversions by the sum of the frequencies of the bases in question. Substitution types were then weighted in the ratio of their expected to observed occurrence.

The Mickevich-Farris index (Mickevich and Farris 1981) was calculated to assess congruence between the cytochrome *b* and 16S rRNA data under a range of weighting strategies with PAUP. In this method, the number of extra steps (additional character conflict) forced on the combined data sets was determined and divided by the length of the combined mp tree. Thus, the optimal weighting strategy for analysis of the combined data was determined.

2.3 Results

The primers Sar and Sbr reliably amplified a 16S rRNA fragment for all galaxioid species included in this study. Of the three light-strand primers used for PCR amplification of cytochrome *b*, the L14724 primer of Meyer *et al.* (1990) was the most reliable for galaxioids. This primer, with H15149 (Kocher *et al.* 1989), consistently produced strong, clean amplifications under stringent reaction conditions (55° C annealing). Good quality sequence data were obtained with all extraction, purification and sequencing methods trialed in this study. A single purified PCR product yielded identical results for each sequencing method, indicating that each technique is reliable. For relatively quick, cost-efficient manual sequencing of galaxioid mtDNA, the chelex extraction, *Wizard* PCR purification and *fmol* sequencing protocols (outlined above) are recommended. Alternatively, the *fmol* method may be replaced with automated sequencing for very quick and reliable, albeit more expensive, results.

Galaxioid sequences were compared with sequences from EMBL and GenBank molecular databases. Without exception, the mtDNA sequences reported in this study were most similar to the corresponding mitochondrial cytochrome *b* and 16S rRNA gene sequences of other teleost fish. The complete cytochrome *b* sequence data set is presented in Table 2.3. Galaxiine 16S rRNA sequences are presented in Table 2.8, while galaxioid 16S rRNA data are shown in Table 2.11.

2.3.1 Cytochrome *b* gene phylogeny

Intraspecific variation and phylogeography

Cytochrome *b* sequences were obtained from more than one individual of some taxa. Identical sequences were obtained for *Galaxias brevipinnis* samples from south-east Tasmania (Snug River) and western Tasmania (Lake Mackintosh). Similarly, no variation was detected between two *G. truttaceus* from Allens Creek in south-east Tasmania. However, considerable genetic variation was detected between isolated populations of *G. zebratus* and *G. maculatus*.

Galaxias zebratus populations

Five cytochrome *b* sequences representing the known geographic range of *G. zebratus* (Fig. 4.2) were analysed. Pairwise sequence divergences (Table 2.4) ranged from 0.33% (a single substitution) between the Kouga and Krom sequences to 13.8% (38 observed substitutions) between the Olifants and Noetsie sequences. The mean interpopulational divergence was 9.9%, or 11.4% if the Kouga and Krom Rivers are treated as a single population. The Noetsie River genotype differed from the Kouga-Krom River sequences by a mean of 6.0%. Sequence divergences were similar when calculated with the Jukes-Cantor algorithm.

Table 2.3 Partial sequences of the mitochondrial cytochrome *b* gene (L-strand) from 33 galaxioids, representing 27 species. Dots indicate matches with the *Galaxias truttaceus* sequence. Inferred amino acid substitutions are represented by an asterisk, and numbers refer to the corresponding positions in human mtDNA.

	Gly	Ser	Leu	Leu	Gly	Leu	Cys	Leu	Ala*	Ser*	Gln	Ile*	Leu*	Thr	Gly	Leu	Phe	Leu
Galaxias truttaceus	GGC	TCC	CTG	CTA	GGG	CTC	TGC	TTG	GCA	AGC	CAA	ATC	CTC	ACC	GGA	TTG	TTT	TTA
Galaxias auratus	.	. .	. .	. G	. .		. . .	.	. .	. .	. .	. .	. .	. .	. .	. . .	. . .	. . .
Galaxias maculatus (Tasmania)	. A	. . T	. C	. T	C	. . T	.	. . .	. T	. .	. G	. . .	T A	. G	. . .	C T	. .	C . T
Galaxias maculatus (WFalkland)	A	.	. .	. . C	C	. . T	.		. C	. T	. . G	. . .	. . T	. G	. . .	C	. C	C . T
Galaxias maculatus (Saunders)	. A	. .	. A	. C	. C	. T	. . .		C	. . T	. A		. T	. A	. G	C .	. C	C . T
Galaxias brevipinnis	A	. . .	T	. .	A	. A	. . .	. . .	. .	.	. .	. .	. T	. A	. .	. .	.	C . C
Galaxias vulgaris	. A	.	. T	G	.	T A	. . .		. .	. .	G	.	T	. G	G	. .	. . .	C . T
Galaxias johnstoni	. A		. T	G	A	A	. .	A		C	. .	.	. . T	A	. .	. .		C C
Galaxias fontanus	A	T	.	. . G		. . G	. . .	.	. .	. C .	.	. .	T	A		. . A	. . .	C . C
Galaxias paucispondylus	G	. .	T	. G	. . A	A	. . .	.	.	. . .	. . G	.	. T	. A	.	. A	. .	C C
Galaxias fasciatus	.	. . .	T	. T	.	.	. . .	. . A	.	. . .	. .	G	T	G	. . .	C		C T
Galaxias argenteus	. G	T	. T	C	. .			. .	. . .	. . .	. .	. . .	. . T	. . A		. . A	. .	C T
Galaxias parvus	. .	.	. T	G	. A	. . .	. . .	C A	ATT	. . .	. .	. T	. A	. . A	. . C	. . A	. .	C . T
Galaxias olidus		.	. T	. G	. .	. . .	. .	. .	. . .	. T	.	. .	. A	. A	. . T	. A	C	C . T
Galaxias zebratus (Olifants)	G	. T	T . A	. T	. A	. . .	.	. . A	.	T	. . .		T A	. A	. . .	C T		. .
Galaxias zebratus (Eerste)	. T	. T	T	. T	. . A	. . .	.	. A	.	T	.	T	T A	. A	. .	C T		. .
Galaxias zebratus (Noetsie)		. T	T	. T	. . A	. .	. .	. . A	.	T	.	. . T	T A	. . G	. .	C . T		
Galaxias zebratus (Kouga)		. . T	T	. T	A	. .	. . .	. A	.	. . T	. .	. . T	T A	. A	.	C . T	. .	.
Galaxias zebratus (Krom)	. .	. T	. T	. . T	A	. .	. . .	A		. . T	.	. . T	T . A	. A	.	C . T	. .	. . .
Galaxias cleaven	. .	T	. T	. G	. . C	.	. .	. A	. .	. . T	.	. . .	T	. . T	. .	C T	. C	C . T
Neochanna apoda	.	. . .	. C	. . C	. C	. .	. . .	. A	G	. . .	. .	. .	. . .	A	.	. . C	C	C . T
Neochanna burrowsius	. .	. . .	T	. T	. . C	. . .	.	. . .	. .	CT	. . G	. . .	. .	. . G	. . .	C A	. . C	C T
Paragalaxias mesotes	.		. . T	T . .	A	.	. . .	A		GCG		. . T	. . A	. A	. T	. . A	.	C . T
Paragalaxias julianus	.	. . .	C	T . .	.	.	.	.	.	GCG		. . T	. A	. A	. C	. . A		C . T
Galaxiella munda	. A	. . T	T . A	. . T	A	. . A	. . .	A	. C	. T	. .	. T	. T	A	. . .	A	. .	. .
Galaxiella nigrostnata	.	. . T	T . A	. . T	.	. . A	. . .	C A	C	. . .	. . G	T	. . T	. A	. C	C . A	. .	. . .
Galaxiella pusilla	. . T	T	. A	. . .	. A	T	T	. . .	C	TCT	. .	T	. ' T	. A	.	. . A		. . .
Brachygalaxias bullocki	. . G	T	. C	C	. . T	T	. . T	. A	. . C	. .	. .	. T	T	. . T	. .	C C	. .	C . T
Nesogalaxias neocaledonicus	. A		. . T	. . G	A	. . G	. .	A	. .	.	. . G	.	. T	T	. . G	.	. . .	C C
Aplochiton zebra	. . .	. . T	. C	. .		A	. . .	. .	. G	. .	G	G . T	. T	. . G	. .	. . A	.	C C
Lovettia sealii	.	. . T	T . A	. T	. . C	. .	. .	. A	. C	.	. . .	. T	.	. . .	. .	. A	. . .	. . .
Retropinna tasmanica	. . G	. . .	. A	. . G	. A	C . A	. T	. A	. . C	. CT	. . G	. T	. . T	.	. . C	C A	. . C	C G
Lepidogalaxias salamandroides	? ? ?	? ? ?	? ? ?	? ? ?	? ? ?	? T	. . ?	? . T	ATC	. TT	. . .	A . T	. . .	G	C . A	. .	C	T
	14850																	

	Ala	Met	His	Tyr	Thr	Ser	Asp	Ile	Ser*	Thr*	Ala	Phe	Ser	Ser	Val	Thr*	His	Ile
Galaxias truttaceus	GCT	ATA	CAC	TAT	ACC	TCC	GAC	ATT	TCT	ACT	GCT	TTT	TCC	TCG	GTT	ACT	CAC	ATC
Galaxias auratus	. .	.	.	. .	.	.	.	. .	. .	. C	. .	. .	. .	. .	. .	. .	. T	. . .
Galaxias maculatus (Tasmania)	. .	G	.	. . .	. . T	. T	. . T	. C	. . .	. . .	. . A	. . .	T	. . A	. . C	. A	. .	. . .
Galaxias maculatus (WFalkland)	. .	G	. .	. . C	.	. T	. . .	. . .	. C	. . C	. . G	. . C	.	.	. . .	. C	. .	. . .
Galaxias maculatus (Saunders)	.	G	. .	. C	. .	. . T	. .	. . .	. . C	. C	. . A	. C	. T	A	. . .	. C	.	. . .
Galaxias brevipinnis	. .	. . .	. T	. . C	. . .	. . G	. . .	. . C	. . .	. . C	. . C	. . .	. . T	. T	. . C	. C	. . T	. . T
Galaxias vulgaris	. . .	. . .	.	. .	. . .	G	. . .	. . C	. . .	. C	. C	. . .	. . T	. . T	. . C	. . .	. T	. . T
Galaxias johnstoni	. .	. . .	. .	. . C	. . .	.	. . .	. . C	. . .	. C	. . C	. . .	. . T	. . T	. . C	. . C	. T	. . T
Galaxias fontanus	. .	. .	. . .	. . .	. . .	.	. . .	. . C	. . .	. C	. . C	. . .	. T	. . T	. . .	. . C	. . .	. . T
Galaxias paucispondylus	. .	. . G	.	. . C	. .	. .	. . .	. . C	. . C	. . C	. . .	. . .	. . .	. . T	. C	. . C	. . T	. . T
Galaxias fasciatus	.	. G	. .	. C	. . T	. T	. . .	. C	. C	. . .	. C	. . .	. . T	. T	. C	. . C	. . .	. . T
Galaxias argenteus	. . C	. . .	. . T	. . C	. T	. . T	. . .	. . C	. .	. . C	. . C	. . C	. T	. . T	. . A	. . C	. . T	. . T
Galaxias parvus	. . C	. G	. . .	. . C	. A	. T	.	. . C	. . C	. .	. . C	. . .	. T	. . T	. .	. . C	. .	. . .
Galaxias olidus	. C	. . .	. . .	. C	. . T	. . T	. . .	. . C	. . C	. . .	. . .	. . .	. A	. . T	. . .	. . C	. T	. T
Galaxias zebratus (Olifants)	. . A	. . .	. .	. .	. . T	. A	. . .	. .	. . A	. .	. . .	. . .	. . T	. . T	. . .	. . C	. T	. T
Galaxias zebratus (Eerste)	. . .	. . .		.	. C	. . T	. . A	. . .	.	A	. . .	. . .	. . .	A	. . C	.	. . C	.	. . T
Galaxias zebratus (Noetsie)	. C	. . .	. . .	. . .	.	T	. A	. . T	. .	. A	. .	. .	. . .	. T	. T	. .	. . C	.	. . ?
Galaxias zebratus (Kouga)	C	. . .	. .	C	. . T	. . G	. . T	. .	. .	. . A	. .	. . .	. . .	. . T	. . T	. . .	C	. .	.
Galaxias zebratus (Krom)	. . C	. . .	. . .	. . .	. . T	. . G	. . T	.	. .	. . A	. . .	. . .	. . .	. . T	. . T	. . .	C	. .	. . .
Galaxias cleaven	. . .	. .	T	.	. . T	.	. .	. . C	. . C	. .	. .	C	C	. .	. . T	. C	. C	.	. . T
Neochanna apoda		. . .	T	. C	. T	. . T	. . .	. C	A	. . .	. . C	. . .	. .	.	. T	C	. . A	. T	. .
Neochanna burrowsius	. C	. . .	. .	. . .	. T	. . T	. .	C	. G	. . C	. . C	. C	.	T	. . C	. C	. . T	. .
Paragalaxias mesotes	.	. .	. . T	. . C	. T	. T	. . T	. C	C	. .	. C	. .	. T	. T	. . .	. . C	. . .	. . .
Paragalaxias julianus	. .	. .	. T	. . C	. T	T	. T		C	. .	. . C		. .	T	. . C	. C	. . .	. .
Galaxiella munda		. .	T	.	T	. .	. . T			. . A	. C	. .	T	. A	. A	G . C	. T	. T
Galaxiella nigrostnata	.	.	. . T	. . C	. .	. . T	. T	. .	. .	A	.	. . .		A	. . A	. A	T	. . T
Galaxiella pusilla	. A	. . .	.	.	. T	. T	. . T	.	.	. . A	. . C	.	. .	A	. .	G . .	. . T	. . T
Brachygalaxias bullocki	.	. .	. .	. C	. .	. . T	T	C	.	. . .	. . .	. C	. T	. T	. A	. . G	. . T	. . T
Nesogalaxias neocaledonicus		. . .	. .	. . .	. . .	. G	. . .	C	.	. . .	. . C	.	. . T	. T	. . C	. .	. T	. . T
Aplochiton zebra	. C	. G	. .	. . C	.	. .	. T		.	. . C	. . C	. . .	. . T	. . C	. . C	. . C	. T	. . T
Lovettia sealii	. .	. . .	. .	. . C	. . T	. .	. T	. C	. . C	. . C	. . C	. . C	. A	. . C	. G	. . A	. . .	. . .
Retropinna tasmanica	. C	. G	. .	. . .	. . G	. . T	. .	. . C	. . .	. A	. . A	. . .		G	A	. . G	G . C	. . .	. . .
Lepidogalaxias salamandroides	. C	.	. . .	. . C	. . A	A	. . T	C	A . A	. A	. .	. C	. A	T	.	G A	T	. . .
	14900																14950	

Table 2.3-continued.

	Cys	Arg	Asp	Val	Ser*	Tyr*	Gly	Trp	Leu	Ile	Arg	Asn*	Met*	His	Ala	Asn	Gly	Aln
Galaxias truttaceus	TGC	CGA	GAC	GTC	AGC	TAC	GGG	TGA	CTA	ATC	CGG	AAC	ATG	CAC	GCT	AAT	GGC	GCA
Galaxias auratus	.	.	. . .	. .	. .	. . .	. .	. . .	. . .	. . .	.	. T	. .	.	. . .	. .	. .	. .
Galaxias maculatus (Tasmania)	. T	. .	. . T	. A	. .	. .	. C	. . .	T . G	. . .	. . .	. .	.	. . .	. .	. C	. A	. . .
Galaxias maculatus (WFalkland)	. . T	. G	. . .	.	. . .	. .	C	G	T G	. .	. . T	. .	. .	. .	. . C	. C	. . G	. . .
Galaxias maculatus (Saunders)	. T	. G	. .	.	. .	. .	. . C	. G	T G		. .	. .	. .	. .	. C	C .	G	. . .
Galaxias brevipinnis	.	. . .	.	.	. .	. . .	. . .	. A	. .	. . G	. T	. A	. .	. .	. . T	. .	.	.
Galaxias vulgaris	.	. .	.	.	.	. .	. .		. .	T	. . A	. .	.	.	. T	. .	.	.
Galaxias johnstoni	. .	. .	.	. T	. .	.	.	.	G	. T	A	. . T	.	.	. T	. . .	. .	.
Galaxias fontanus	. .	. . G	. T	. .	. .	. .	. . .	G	.	. . T	. A	. T	.	.	. T	C	. T	.
Galaxias paucispondylus	. .	. G	.	. . T	. . .	. .	. A	G	. G	. T	. . T	. T	. A	. T	. C	. C	. .	. .
Galaxias fasciatus	. .	G	. .	. A	. . T	.	. T	. .	T .	.	. A	. . T	. .	. T	. C	C		
Galaxias argenteus	. .			. . A	. T	.	.	. .	. .	. . T	. A	. .	.	A	. T	. .	. C	. . .
Galaxias parvus	. .	.	.	. . T	C .	.	. .	. .	T . .	. T	. . A	. T	. A	. T	. . .	. . .	. .	. . .
Galaxias olidus	. .	. . .	. . .		. .	. .	. .	. .	. .	T	A	. .	.	. . T	. . .	. . .	. .	. .
Galaxias zebratus (Olifants)	T	. . T	. T	. . A	. . .	. .	.	. . G	. .	. T	. . A	. . T	. . .	. T	.	. . C	. .	
Galaxias zebratus (Eerste)	. . .	.	T	. . G	. A	T	A		.	. . T	. . T	. T	. .	T	. .	. C	.	.
Galaxias zebratus (Noetsie)	. .	. T	. T	. . A	T		.	. . .	. T	. . T	T	. .	.	.	. . .	.	. . .	. .
Galaxias zebratus (Kouga)	. . .	T	. T	. G	. . T	. .	.	.	. .	. . T	. . C	. . T	. .	. . .	. C	. C	. .	. .
Galaxias zebratus (Krom)	. . .	. T	. . T	. G	. . T	. .	. . .	. .	.	T	. . C	T	. .	. .	. . C	. C	. .	. .
Galaxias cleaveri	.	. T	. .	. .	. .	. . .	. C	. .	T .	. . T	A	. .	. .	. .	. . C	C	. .	
Neochanna apoda	. .	T	.	. .		. . T	C	. .	. . .	. T	. . .	. . T	. .	. T	. C	. C	. .	.
Neochanna burrowsius	. .	. . T	. . .	. .	. .	. T	. .	. . G	. .	. T	. . A	.	. .		. C	. . C	. T	. .
Paragalaxias mesotes	. .	. .	. . .	T	. . .	.	. A	. . .	. .	. . T	. . A	. T	. .	T	.	. . C	. . .	. .
Paragalaxias julianus	. .	. .	. T	T	. T	. .	A	. .	. .	T	. . A	.	. .	T	. .	. C	. . .	.
Galaxiella munda	. .	. . C	. T	. T	G . T	. T	. . C		. . T	.	. . T	. T	. A	. T	. .	. .	A	.
Galaxiella nigrostriata	. .	. .	. T	.	. .	. .	. C	. .	. .	. .	. T	. . T	. .	. A	. .	. C	. A	.
Galaxiella pusilla	. .	. .	.	. . T	G .	. . T	. . C	. .	. T	. .	. . C	. .	. .	. A	. .	. C	. . A	. . .
Brachygalaxias bullocki	.	. T	T	. .	. .	.	. C	. .	. .	. . T	. A	. G .	. .	.	A	.	. G	. C
Nesogalaxias neocaledonicus	.		.	. .	. .	. .	A	. G	. . G	. . T	. .	. T	. .	. . T	. .	.	.	. .
Aplochiton zebra	. .	. .	. . T	. . T	. .	. .	. C	G	. .	.	.	G T	. .	. . T	. C	. .	.	. .
Lovettia sealii	. . .	. . T	. .	. T	G A G	.	. C	. . G	T . .	. . T	A	. T	? ? ?	? ? ?	. . C	. C	. .	? ?
Retropinna tasmanica	.	. G	. T	. . G	A .	. .	. C	. G	G	. .	. . C	. . .	. .	. .	. T	. . C	. . C	A .
Lepidogalaxias salamandroides	. .	. G	.	. . .	T C .	A . .	. . A	. .	T	T	. . C	G	. . T	. . T	. . C	. C	.	.

15000

	Ser	Phe*	Phe	Phe	Ile	Cys	Ile	Tyr	Met*	His	Ile	Gly*	Arg	Gly	Leu	Tyr	Tyr	Gly
Galaxias truttaceus	TCT	TTT	TTC	TTC	ATT	TGC	ATC	TAT	ATA	CAT	ATC	GGC	CGG	GGC	CTT	TAT	TAC	GGC
Galaxias auratus	. . .	. C	. . .	. . .	. . .	. .	. . .	. .	. .	. .	. . .	. .	. . .	. . .	. .	. .	. .	. .
Galaxias maculatus (Tasmania)	. . .	. . C	. T	. . .	. C	T	. T	. . .	. . G	. . C	. . .	. G	. A	. . T	T . G	. . C	. . T	. . .
Galaxias maculatus (WFalkland)	. .	. . .	.	. . .	. C	. . T	. T	. . .	G	. C	. .	. . A	. . A	. G	. . G	. . C	. . .	. . .
Galaxias maculatus (Saunders)	. .	. . .	. . .	. . .	. . C	. . T	. . T	. .	C	. C	. . .	. . A	. A	. G	. . G	. C	. . .	. . .
Galaxias brevipinnis	. .	. . C	. T	. . .	. C	. .	. . T	. C	. G	. . C	. . T	. . A	. . T	. A	?	. . .	. . .	. G
Galaxias vulgaris	. . .	. . C	. T	. . T	. . .	. . T	. . T	. .	. . G	. . C	. . T	. . A	. A	. . A	. .	. . .	. T	. G
Galaxias johnstoni	. .	. C	. . T	. . .	. . C	. . .	. . T	. .	. . G	. . C	. . T	. . A	. . A	. . A	. G	. .	. . T	. G
Galaxias fontanus	. . .	C	. . T	. . T	. . C	. T	. T	. . .	. . .	. C	. . T	. G	. . .	. . A	. . .	. . .	. . T	. . A
Galaxias paucispondylus	. . .	. C	. . T	. . .	. . C	. . .	. . T	. .	. . G	. . C	. . .	. . A	. A	. . A	. .	. . .	. T	. . G
Galaxias fasciatus	.	. C	. . .	. .	. . C	. . .	. . .	. .	. . G	. . C	. . .	. . G	. A	. A	. .	. . .	. .	. . G
Galaxias argenteus	. . .	. . C	. .	. .	. . C	. . T	. . T	. . .	. . G	. . C	.	. . A	. A	. A	. C	. .	. T	. . G
Galaxias parvus	. . A	C	. .	. .	. . C	. . .	. .	. C	. . .	. C	.	. . A	. A	. T	. . .	. . C	. .	. . G
Galaxias olidus	. . A	C	. . T	. . T	. . C	. . T	. .	. C	C . G	. . C	. . .	. A	. . A	.	. .	. . C	. . .	. . T
Galaxias zebratus (Olifants)	. . .	. C	. T	. . T	. . .	. . T	. T	. . .	. . .	. . C	. . T	. . A	. A	. A	. A	.	. . .	. . G
Galaxias zebratus (Eerste)	.	. . .	. .	. . T	. . .	. T	. T	. . .	T .	. . .	. . T	. . A	. A	. . A	. .	. . .	. . T	. . .
Galaxias zebratus (Noetsie)	. .	. . .	. . .	. . T	. . C	. . T	. . T	. . .	T . .	. . .	. T	. . A	A	. . A	.	. . .	. . .	A
Galaxias zebratus (Kouga)	. .	. .	. . .	. . T	. .	. T	. . T	. . .	C G	.	. . T	. . A	. . A	. . A	. . .	. .	. . .	. . A
Galaxias zebratus (Krom)	. . .	. . .	. . .	. . T	. . .	. T	. . T	. . .	C . G	. .	. T	. A	. A	. . A	. . .	. . .	. . .	. . A
Galaxias cleaveri	. C	C	. . .	. . T	. . C	. . .	. . T	.	. . G	. . C	. . .	. A	. .	. . G	. . .	. C	. . .	. . .
Neochanna apoda	. . . C	C	. T	. . .	. . C	.	. . .	. . C	C . G	. C	. . .	A	A	. . G	. .	.	. T	. . G
Neochanna burrowsius	. . .	. C	. . T	. .	. . C	. T	. .	. C	.	C	. . .	. . G	. . A	A	. .	. .	. T	. G
Paragalaxias mesotes	. . .	. . .	. . .	. .	. . T	. .	. . T	. T	. . .	. .	. . C	. . T	. A	. .	. .	. C	.	. . A
Paragalaxias julianus	. .	. .	. . .	. T	. C	. T	. T	. .	G	. .	. T	A	. . A	. .	.	. C	T	. . T
Galaxiella munda	.	. C	. . T	.	. .	. T	. T		T .	.	. T	A	. . A	A	. .	. C	. .	T
Galaxiella nigrostriata	. . A	. C	. T	. T	. .	. T		.	. G	. C	T	. . G	. A	. . T	.	. C	. T	. G
Galaxiella pusilla		C	.	T	. . .	. . T	.	C	T .	. . .	. .	. . A	. .	. .	. .	. .	. .	. .
Brachygalaxias bullocki	A	. C	. . T	. . T	. . C	. .	. . .	. . .	G	. .	. . T	. . A	. .	. .	. .	. C	. . T	. .
Nesogalaxias neocaledonicus	.	. C	. . T	. T	. . C	. .	T	C	. . G	. C	. . T	A	. .	. . A	. . .	. . .	. .	. . A
Aplochiton zebra	. . C	.	. . .	.	. C	. . T	T	. .	. G	C	.	. . .	. . A	. G	. . .	. . .	. . T	. . .
Lovettia sealii	. . C	. C	. T	. . T	. .	. . T	. T	. . C	C . T	.	. . T	. . .	. . T	. A	. . .	.	. . T	. . .
Retropinna tasmanica	. . A	. . C	. . T	. . T	. C	. .	. T	. C	C C	. C	. . T	. . .	. . A	. . T	. G	. C	. .	. G
Lepidogalaxias salamandroides	. A		.	. . .	. T	. C		T	. .	.	C	. . .	C .	A	T . G	. C	T	. G

15050

Table 2.3-continued.

	Ser	Tyr*	Leu*	Tyr	Lys	Glu	Thr	Trp	Thr*	Ile*	Gly	Val*	Ile*	Leu	Leu	Leu	Leu	Val*
Galaxias truttaceus	TCC	TAC	CTA	TAT	AAG	GAG	ACT	TGG	ACT	ATT	GGG	GTC	ATC	CTT	CTT	CTT	CTC	GTC
Galaxias auratus	...	...	...	...	..A	..	...	...	..	..	..	...	...	...	...	...	...	...
Galaxias maculatus (Tasmania)	..T	..T	..G	..C	.A	..	C	.A	.C	..C	.A	.A	..T	..C	..C	..C	..T	..
Galaxias maculatus (WFalkland)	..	.T	..C	..	..		..C	A	C	.C	..	.G	G.	A	C	.A	T	T
Galaxias maculatus (Saunders)	..	..T	..C	..	..		.C	.A	.C	.C	.	..G	G.	..A	..C	T A	T	T
Galaxias brevipinnis	..T	..	.C	..	.A	...	C	.	.	.	.T	..A	G..	.?	? C	...	..T	..G
Galaxias vulgaris	T	...	.C	...	...	...	C	..A	.C	...	..T	..T	G..	...	C	..A	.T	..G
Galaxias johnstoni	...	...	..	...	..	.	..C	..A	.	...	..T	..A	G..	.	...	...	.T	.G
Galaxias fontanus	..T	...	..C	..	..A	...	.C	.	...	...	..T	.T	..T	.C	C	.C	.T	.A
Galaxias paucispondylus	..T	..	.C	.	.A	..	.C	..A		...	..C	...	G..	..	...	..G	.T	.A
Galaxias fasciatus	.A	...	..	...	.	..	..C	..A	.C	C	..A	...	G A	...	.A	..G	..	.
Galaxias argenteus	.	...	C	..C	A	.	..C	..A	GC	C	..A	..G	G.A	..	T.A	..A	.	.
Galaxias parvus	.T	..T	..T	..C	.A	...	..C	..A	.A.	...	...	.A	. .	C	.A	..C	.G	.A
Galaxias olidus	...	.	..T	...	..A	..A	.C	.A	A	...	...	..T	G G	.C	. C	. C	..	.A
Galaxias zebratus (Olifants)	..T	.T	..T	...	..A	...	.C	.A	.AC	...	..A	..G	G.	...	T A	..	.T	.A
Galaxias zebratus (Eerste)	.A	...	.T	..	.A	..	..A	..A	A	. .	.A	..A	G T	..G	T A	..	T	.
Galaxias zebratus (Noetsie)	.	...	..T	.C	.A	.A	.A	..A	A		.A	..A	G .	..	T G	. .	..T	.
Galaxias zebratus (Kouga)	.T	..T	...	. .	.A	..A	A	..A	A.	...	.A	..A	G ,		T G	. .	.T	..
Galaxias zebratus (Krom)	.T	..T	..	...	..A	..A	..A	..A	A.	..	.A	..A	G . .		T G	..	.T	.
Galaxias cleaven	.	...	..T	...	...	. .	.C	..A	.AC	C	...	.T	G A	.	...	...	T	..A
Neochanna apoda	...	..	..C	..	..A	..A	..C	..A	AC	..	..C	..A	.T	C	A	.C	..	..G
Neochanna burrowsius	...	..	..T	..	.A	..A	..C	..A	AC	...	..C	..A	G T	A	T.A	.C	.T	.A
Paragalaxias mesotes	..T	...	..C	..C	...	..	.C	.A	. C	..	..A	.	A G A	.	. A	..	..	..A
Paragalaxias julianus	.T	...	.T	..C	.A	. A	.C	. A	C	..	A	. .	G A	C	C	...	...	..A
Galaxiella munda	. T	..	T .	..C	A	. A	..C	..A	A	.	.T	..T	G A	T A	T A	...	T	A
Galaxiella nigrostriata	A	..T	...	...	.	A	..A	..A	. A	GC	C	T	T	G.A	T A	.A	. .	..T	A
Galaxiella pusilla	.T	..T	T..	...	.	A	A	..C	.A	A.	G .	..T	T	G A	T A	G	..	.T	..A
Brachygalaxias bullocki	.T	. .	A.T	..C	...	...	..A	..A	A	...	.	T	. A	G A	T A	G	C	..	...
Nesogalaxias neocaledonicus	.	.	.	.	...	...	.C	..A		...	..T	.	A G	G	.	C	..A	...	..G
Aplochiton zebra	.A	..T	T	..C	.A	. A	..C	..A	AC	C	.	.	G G	T	..G	...	..C	..T	.T
Lovettia sealii	..T	..T	..T	...	..A	..A	..C	. A	.	...	. A	...	G T	.	..C	..C	. A	. T
Retropinna tasmanica	. T	.T.	..G	..C	...	...	...	A	TTC	T A	A	CG	G.T	..G	. C	..C	. T	A
Lepidogalaxias salamandroides	..T	...	T..	...	..A	...	...	..A	CT	G.G	..C	..A	G.A	..A	T A	...	T A	A.A

15100

	Met	Met*	Thr	Ala	Phe	Val	Gly	Tyr	Val	Leu	Pro
Galaxias truttaceus	ATG	ATG	ACT	GCC	TTT	GTG	GGC	TAT	GTC	CTC	CCC
Galaxias auratus	...	...	..	..	...	...	...	..?	???	???	???
Galaxias maculatus (Tasmania)	...	...	..C	..T	...	..T	. G	..C	. T	.	...
Galaxias maculatus (WFalkland)	...	...	...	..T	..C	..T	..A	..C	..	...	...
Galaxias maculatus (Saunders)	..A	...	...	..T	..C	..T	..A	..C	...	...	...
Galaxias vulgaris	...	..A	...	...	.C	..C	..A	...	.	.	..T
Galaxias johnstoni	..A	...	...	...	.C	..T	..G	...	..	.	...
Galaxias fontanus	...	.A	...	..T	..C	..T	..G	..	..T	.	..A
Galaxias paucispondylus	...	...	...	...	..C	..T	..A	...	..T	...	...
Galaxias fasciatus	...	...	...	...	.	..T	..T	..C	.	..T	.
Galaxias argenteus	.	...	...	...	. C	..T	.	..C	.	..T	...
Galaxias parvus	..A	...	..A	..T	...	. T	.G	...	.	.	
Galaxias olidus	. A	..A	A	...	...	T	...	..	..	...	...
Galaxias zebratus (Olifants)	..A	..A	...	..T	...	. T	..A	.	..T	..	. T
Galaxias zebratus (Eerste)	..A	. A	..A	..T	...	.	...	..	. T	..	.T
Galaxias zebratus (Noetsie)	..A	G.A	..A	..T	...	..A	..A	..C	.T	T G	..T
Galaxias zebratus (Kouga)	...	..A	..A	..T	...	..A	..A	. C	..T	T G	..T
Galaxias zebratus (Krom)	..	..A	..A	..T	...	. A	..A	..C	.T	T.G	..T
Galaxias cleaven	...	...	..C	...	..C	..T	..G	...	..T	. T	..T
Neochanna apoda	..A	...	..C	..T	..C	..A	..G	..C	. T	...	...
Neochanna burrowsius	...	...	..C	..T	..C	...	..G	...	...	. .	...
Paragalaxias mesotes	. A	...	..A	...	...	..T	..G	...	..A	...	...
Paragalaxias julianus	..A	..A	..A	..	...	..T	..G	...	..G	...	..
Galaxiella munda	..A	..A	...	..T	...	..T	..	. C	..T	T.A	..
Galaxiella nigrostriata	..A	...	...	..T	...	...	...	..C	. A	T A	..
Galaxiella pusilla	..A	..A	...	..T	...	.	.	. .	T	T.G	..A
Brachygalaxias bullocki	..A	..A	..C	...	...	. A	..	. .	. T	. T	...
Nesogalaxias neocaledonicus	...	.	...	...	..C	..C	..G	. .	. T	..	...
Aplochiton zebra	..A	..A	...	...	...	..C	..	...	. T	.T	...
Lovettia sealii	...	..A	...	...	...	...	...	...	. T	..T	. T
Retropinna tasmanica	...	...	...	...	..C	..T	..G	..C	A	.T	.
Lepidogalaxias salamandroides	. A	..A	..A	...	..C	..?	..?	???	???	???	???

Table 2.4 Percentage sequence divergences (above diagonal) and observed nucleotide substitutions (below diagonal) between *Galaxias zebratus* cytochrome *b* sequences. Numbers in brackets are observed transversion substitutions. Divergence values were calculated with the maximum likelihood algorithm of Felsenstein (1993).

Taxon	Olifants	Eerste	Noetsie	Kouga	Krom
G. zebratus (Olifants)	–	12.6	13.8	13.3	12.9
G. zebratus (Eerste)	35 (16)	–	11.4	11.3	11.7
G. zebratus (Noetsie)	38 (12)	24 (8)	–	6.2	5.8
G. zebratus (Kouga)	37 (12)	32 (8)	18 (2)	–	0.3
G. zebratus (Krom)	36 (12)	33 (8)	17 (2)	1 (0)	–

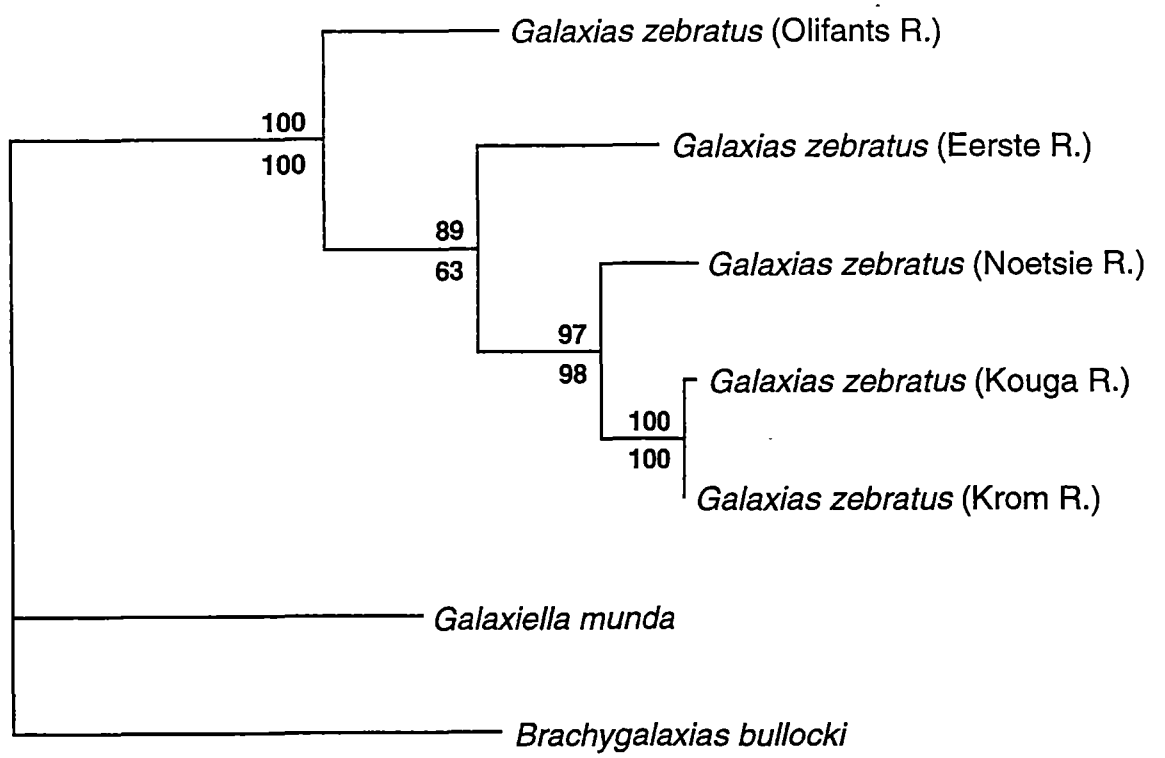

Figure 2.2 Intraspecific phylogeny of *Galaxias zebratus* based on 303 bp of cytochrome *b* sequence. The parsimony analysis was rooted with the outgroups *Galaxiella munda* and *Brachygalaxias bullocki*. A TV/TI ratio of 2:1 was used. Values at branch points indicate bootstrap support (500 resamplings); values above nodes are for parsimony analysis while those below nodes are for distance analysis. Branch lengths correspond to the number of character state changes.

A total of 56 nucleotide sites varied, 51 of which were third codon positions, while four were first positions and one was a second position. Two changes in the amino acid sequence of *G. zebratus* cytochrome *b* are inferred from the sequences. Specifically, a serine residue (position 14966-14968, Table 2.3) is replaced by an asparagine residue in the Eerste River specimen, and a methionine residue (position 15119-15121, Table 2.3) is replaced by a valine residue in the Noetsie River genotype. Of the observed pairwise substitutions, 71% were TIs while 29% were TVs, representing a TI bias of less than 3:1. Based on an analysis of silent sites (third codon positions) only, the Olifants genotype differed from other genotypes by a mean corrected sequence divergence of 43.5%.

For parsimony analyses, tree polarity was determined by the inclusion of *Galaxiella munda* and *Brachygalaxias bullocki* sequences as outgroups. Additional outgroup taxa were initially included but were found to have no effect on the *G. zebratus* tree topology. An exhaustive search found a single most parsimonious (mp) tree, 210 steps in length (Fig. 2.2). In this topology, the Krom-Kouga clade was grouped with the specimen from the Noetsie River to form an eastern clade. In turn these three sequences formed a clade with *G. zebratus* from the Eerste River near Cape Town. The single genotype representing the north-western Olifants river population was placed as basal to the other genotypes. The mp tree was four steps shorter than the next shortest tree (214 steps). Different weighting schemes for TVs and TIs (1:1, 4:1 and 8:1) had no effect on the mp tree topology. Similarly, the topology was not altered by preferential weighting of first and second codon positions over third codon positions. Bootstrapping provided a high level of statistical confidence in the topology, with values of over 90% for three of the four nodes (Fig. 2.2).

Galaxias maculatus populations

A substantial level of intraspecific diversity was also detected between *G. maculatus* isolates. Pairwise sequence divergences were calculated with a TV/TI ratio of 2:1. They ranged from 4.1% (12 substitutions) between the West Falkland and Saunders Island sequences to 19.2% (52 observed substitutions) between the Tasmanian and Saunders Island sequences (Table 2.5). The Tasmanian sequence differed from the two Falklands sequences by a mean of 18.8%. Sequence divergence values were similar when calculated with the Jukes-Cantor algorithm. Based on an analysis of silent sites (third codon positions) only, the Tasmanian genotype differed from the Falkland sequences by a mean corrected sequence divergence of 71.1%. The corrected silent divergence between the Saunders Island and West Falkland sequences was 11.0%.

Table 2.5 Percentage sequence divergences (above diagonal) and observed nucleotide substitutions (below diagonal) between *Galaxias maculatus* cytochrome *b* sequences. Figures in brackets are observed numbers of transversion substitutions. Divergence values were calculated with the maximum likelihood algorithm of Felsenstein (1993).

Taxon	Tasmania	West Falkland	Saunders Island
G. maculatus (Tasmania)	–	18.1	19.5
G. maculatus (West Falkland)	48 (10)	–	4.1
G. maculatus (Saunders Island)	52 (9)	12 (1)	–

A total of 55 nucleotide sites varied, 50 of which were third codon positions, while four were first positions and one was a second position. Two changes in the amino acid sequence of *G. maculatus* cytochrome *b* are inferred from the sequences. Specifically, a methionine residue (position 15032-15034, Table 2.3) is replaced by a threonine residue in the Saunders Island specimen, and a valine residue (position 15098-15100, Table 2.3) is replaced by an isoleucine residue in the Tasmanian genotype. Of the observed pairwise substitutions, 82% were TIs while 18% were TVs, representing an observed TI bias of over 4:1.

Parsimony analysis of the *G. maculatus* cytochrome *b* sequences was conducted with the inclusion of *G. brevipinnis* and *G. truttaceus* as outgroups (Fig. 2.3). The Saunders Island and West Falkland genotypes were grouped with very high bootstrap support (99%), and the three *G. maculatus* sequences formed a clade with similar support (99%).

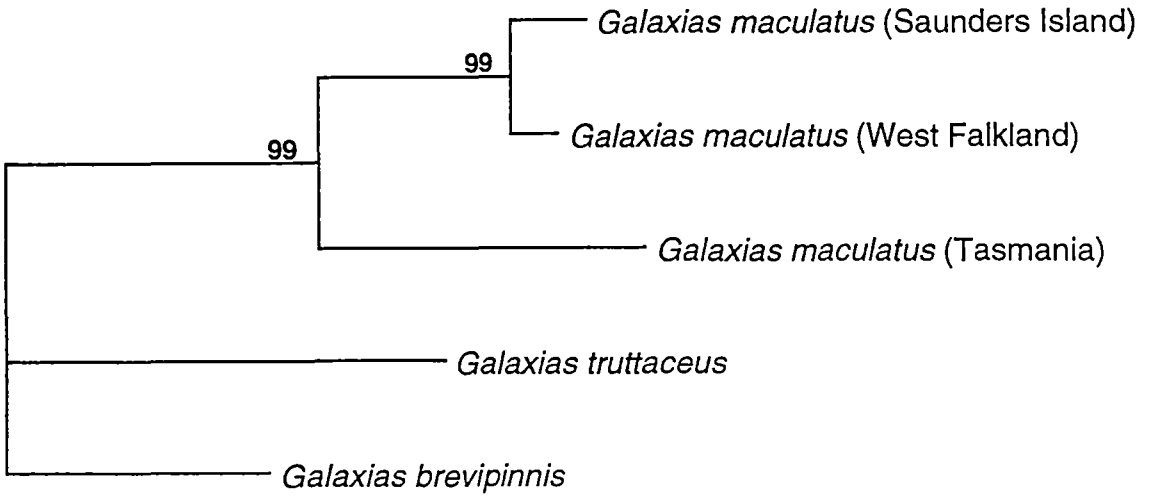

Figure 2.3 Intraspecific phylogeny of *Galaxias maculatus* based on 303 bp of cytochrome *b* sequence. The parsimony analysis was rooted with the outgroups *Galaxias brevipinnis* and *Galaxias truttaceus*. A TV/ TI ratio of 2:1 was used. Values at branch points indicate the percentage of bootstrap replicates supporting the clade directly to the right of the node, based on 500 resamplings of the data. Branch lengths correspond to the number of character state changes.

Interspecific variation

This analysis was initially restricted to 25 galaxiine sequences, representing 23 extant species of the Galaxiinae, and the South American *Aplochiton zebra* as an outgroup. *Galaxias zebratus* and *G. maculatus* were represented by the two most divergent sequences detected within these species (Noetsie and Olifants River *G. zebratus*; Tasmanian and Saunders Island *G. maculatus*). No length variation was detected between these sequences. Of the 303 nucleotide positions sampled, 179 (59%) were invariant, while 124 (41%) were variable. Of the variable sites, 101 (82%) were third codon positions (representing every third position sampled), 20 (16%) were first positions, and three (2%) were second positions. The majority (65%) of the first position substitutions were silent changes at codons encoding for leucine.

Based on all pairwise comparisons of sequences, 70% of the observed substitutions were TIs while 30% were TVs, representing a TI bias of about 2.3. The observed TI bias increased slightly (2.5) when pairwise comparisons were limited to sequences separated by a maximum of 20% corrected sequence divergence. Observed numbers of TIs and TVs were plotted against corrected sequence divergence for pairwise comparisons, excluding those that differed by greater than 20% (Fig. 2.4). Linear relationships were detected between genetic distance and both TIs and TVs.

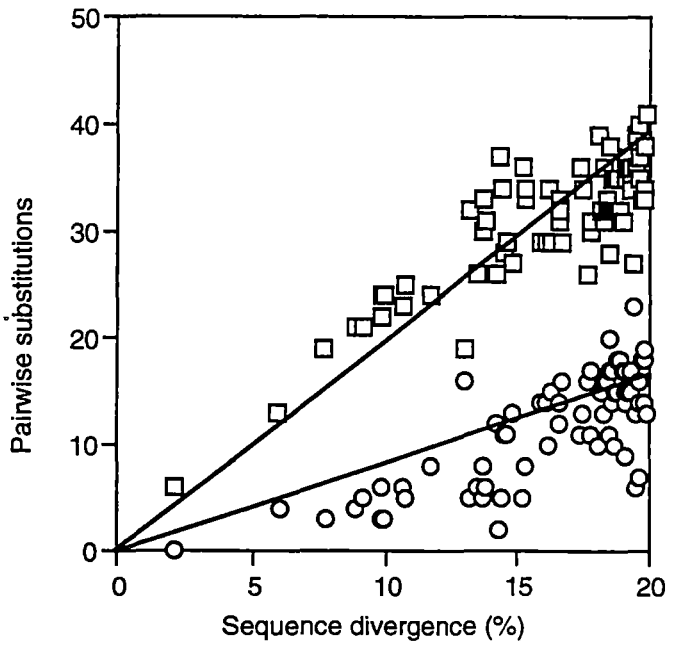

Figure 2.4 Observed TIs (open squares) and TVs (open circles) plotted against pairwise sequence divergence. Divergence values were calculated with the maximum likelihood algorithm of Felsenstein (1993) and are corrected for multiple hits. Pairwise comparisons were limited to taxa that differed by ≤20% sequence divergence to avoid saturation with TIs. Linear regression lines for TIs (r2 = 0.74) and TVs (r2 = 0.60) are shown.

Table 2.6 Percentage sequence divergences (above diagonal) and observed number of nucleotide substitutions (below diagonal) for pairwise comparisons of galaxiid cytochrome *b* sequences. Divergence values were calculated with the maximum likelihood algorithm of Felsenstein (1993).

Taxon	*Gtr*	*Gau*	*Gmt*	*Gmf*	*Gbr*	*Gvu*	*Gjo*	*Gfo*	*Gpc*	*Gfa*	*Gar*	*Gpv*	*Gol*	*Gzo*	*Gzn*	*Gcl*	*Nap*	*Nbu*	*Pme*	*Pju*	*Glm*	*Gln*	*Glp*	*Bbu*	*Nne*	*Aze*
G. truttaceus	–	2.1	27.3	22.7	19.1	20.6	19.3	22.3	22.2	20.0	24.0	24.7	20.9	26.4	25.3	22.1	25.0	25.6	22.9	23.6	30 8	28.8	24.9	26.9	18.8	25.1
G. auratus	6	–	28.0	24.5	19.0	20.1	18.3	20.4	20.0	20.4	22.8	24 8	20.8	25.1	25 4	22.5	24.7	24.3	25.2	25.9	28.1	26.0	23.4	27.6	18.2	24.3
G. maculatus (T)	69	68	–	18.1	24.0	25.3	25.0	24.0	25.2	21.5	23.7	24.8	28.1	24.9	27.8	23.6	23.5	22.3	25.8	24.4	30.1	26.4	32.7	26.6	26.0	22.9
G. maculatus (F)	59	61	49	–	21.5	22.2	22.6	25.4	19.1	20.1	22.2	28.0	25.7	27.5	31.1	18.8	20.9	24.5	27.6	28.0	33.3	29.4	32.7	30.3	23.5	23.0
G. brevipinnis	50	48	61	56	–	9.9	5.9	13.8	10.7	18.6	14.8	20.3	17.8	21.2	27.3	19.8	19.6	19.3	18.6	20.3	24.6	28.1	29.1	25.0	8.8	23.5
G. vulgaris	54	51	64	58	28	–	10.6	13.7	14.3	19.6	16.7	25.9	18.5	20.7	27.6	20.4	20.4	24 0	21.5	22.3	28.5	26.9	30.8	25.9	9.8	23.0
G. johnstoni	51	47	64	59	17	30	–	14.4	9.9	16.8	15.9	19.3	18.9	20.3	26.3	19.2	20.3	19.6	17.5	21.3	23.9	26.9	30.0	24.4	9.1	21.7
G. fontanus	58	52	61	65	38	38	40	–	15.2	23.6	21.4	24.2	20.6	20.4	27.9	21.8	19.5	24.1	21.8	22.6	25.2	28.8	28.0	26.4	15.3	22.7
G. paucispondylus	58	51	64	51	30	40	28	42	–	16.6	16.6	21.0	20.1	20.8	29.4	19.5	19.1	20.0	21.4	22.2	26.4	27.1	30.5	28.1	13.2	20.0
G. fasciatus	53	52	56	53	49	52	45	61	45	–	13.7	20.3	21.0	23.5	26.5	16.3	21.0	19.7	18.5	21.0	29.3	25.5	34.3	26.5	19.2	22.0
G. argenteus	62	57	61	58	40	45	43	56	45	38	–	22.2	18.4	21.0	25.3	18.8	18.7	20.4	19.3	19.8	25.0	25.7	31.1	24.7	20.4	21.6
G. parvus	63	61	63	70	53	66	51	62	55	53	58	–	14.6	23.9	26.5	23.6	22.7	21.0	16.2	20.4	29.9	29.0	31.2	28.1	23 7	25.9
G. olidus	55	53	40	65	47	49	50	54	53	55	49	40	–	21.4	23.4	18.3	17.7	18.5	17 4	15.3	27.6	26.6	26.0	22.9	19.8	23.9
G. zebratus (O)	66	61	63	69	55	54	53	53	54	60	55	61	55	–	13.8	22.0	21.8	24.9	20.0	24.2	21.0	24.9	26.2	28.8	22.5	26.3
G. zebratus (N)	63	61	68	75	67	68	65	68	72	66	64	66	59	38	–	24.7	25.2	28.2	22.1	22.7	20.7	30.3	25.8	26.7	27.6	27.1
G. cleaveri	58	57	61	50	52	54	51	57	52	44	50	61	49	57	62	–	17.8	18.5	20.1	21.7	30.2	29.7	29.8	21.6	19.1	23.7
N. apoda	64	61	60	55	52	54	54	52	51	55	50	59	47	57	64	48	–	13.6	24.4	24.1	30.7	28.9	28.3	28.4	23.0	24.7
N. burrowsius	65	60	58	63	51	62	52	62	53	52	54	55	49	64	71	50	38	–	22.9	23.0	30.2	26.5	32.3	24.1	20.9	24.8
P. mesotes	59	62	65	69	49	56	47	57	56	49	51	44	47	52	56	53	63	59	–	7.6	25.2	26.3	28.2	25.9	20.6	26.4
P. julianus	61	64	62	70	53	58	56	59	58	55	52	54	42	61	57	57	62	59	22	–	25.7	26.4	27.2	25.6	25.0	22.8
G. munda	75	67	73	81	61	70	60	63	66	72	63	72	68	53	52	74	75	74	62	63	–	16.1	14.5	26.6	28.5	26.5
G. nigrostriata	71	63	65	72	68	66	66	70	67	64	64	71	66	61	72	72	71	66	65	65	43	–	21.7	24.6	29.7	23.4
G. pusilla	63	58	78	79	70	74	73	69	74	82	75	75	65	64	63	73	70	78	69	67	39	56	–	26.8	30.8	29.0
B. bullocki	67	66	67	75	62	64	61	65	69	66	63	69	58	70	65	56	71	62	64	63	66	62	66	–	24.4	24.5
N. neocaledonicus	50	47	66	61	25	28	26	42	37	51	54	61	52	58	68	51	60	55	54	64	70	72	74	61	–	24.3
A. zebra	65	61	59	60	61	60	57	59	53	58	57	66	62	66	67	62	64	64	67	59	66	59	71	62	63	–

For interspecific comparisons, corrected sequence divergences ranged from 2.1% between *G. truttaceus* and *G. auratus* to 34.3% between *G. fasciatus* and *Galaxiella pusilla* (Table 2.6). A wide range of values was recorded for intrageneric comparisons. For example, 13.6% divergence was detected within *Neochanna*, 7.6% within *Paragalaxias*, and a mean of 16.6% between species of *Galaxiella*. The New Caledonian *Nesogalaxias neocaledonicus* sequence was separated from that of *G. brevipinnis* by 8.8% divergence. *Galaxias zebratus* (mean divergence from other taxa = 25.0%), *Brachygalaxias bullocki* (26.2%) and *Aplochiton zebra* (24.1%) were each separated from all other taxa by a minimum of 20% corrected sequence divergence. A considerable amount of genetic diversity was detected between members of the genus *Galaxias*, with a mean divergence of 19.9% and a maximum divergence of 29.4% between *G. zebratus* and *G. paucispondylus*. Relatively few pairwise comparisons revealed less than 10% sequence divergence. A mean of 11.4% divergence separated species in the group containing *G. brevipinnis*, *G. vulgaris*, *G. johnstoni* and *G. fontanus*. *Galaxias fasciatus* and *G. argenteus* sequences differed by 13.7%. The Tasmanian mudfish *G. cleaveri* was separated from the New Zealand mudfish genus *Neochanna* by a mean of 18.2% divergence. *Galaxias parvus* and *G. olidus* were separated by 14.6% divergence.

Galaxiine cytochrome b phylogeny

Initially, an attempt was made to assess the phylogenetic information content of the data. With the inclusion of all galaxiine sequences, the length distribution of 10,000 random trees showed a significant skew to the left ($g1$ = -0.54, $P<0.01$) indicating a strong phylogenetic signal in the cytochrome *b* data set (Hillis 1991; Hillis and Huelsenbeck 1992). When all but the most divergent sequences were eliminated, the tree-length distribution was less markedly skewed, but still indicated that the data were not randomised with respect to phylogenetic history ($g1$ = -0.40, $P<0.01$).

Maximum likelihood (ML) methods were used to determine the optimal TI bias for the cytochrome *b* data set. A subset of nine galaxiine taxa (excluding closely related species) was chosen for analysis. The log likelihood of the ML tree was calculated using a range of TI/TV ratios (1:1, 2:1, 3:1, 4:1 and 6:1). The tree with the greatest log likelihood, and hence the optimal TI bias, was achieved with a weighting of 2:1 (Fig 2.5). The log likelihoods produced with TI ratios of 2:1 and 3:1 were considerably greater than those attained with higher and lower ratios, suggesting that they provide a reasonable estimate of the actual TI bias. Because the optimal TI bias also approximated the observed TI bias (about 2.5:1), a TI/TV ratio of 2:1 was chosen for phylogenetic analyses of the cytochrome *b* data set.

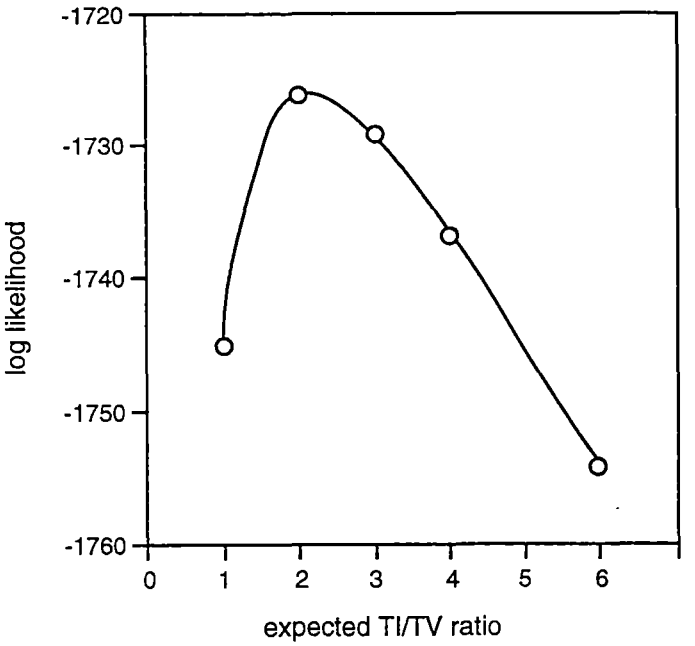

Figure 2.5 Log likelihood values of maximum likelihood trees calculated for a subset of nine galaxiine cytochrome *b* sequences under a range of expected TI/TV ratios. The tree with the highest log likelihood (-1726.4) had an expected TI/TV ratio of 2.

Maximum likelihood analysis of the galaxiine cytochrome *b* data (rooted with *Aplochiton zebra*) recovered a tree with a log likelihood of -3654.2 (Fig 2.6). The ML topology supported the monophyly of the genera *Neochanna*, *Paragalaxias* and *Galaxiella*. Within, *Galaxiella*, *G. munda* and *G. pusilla* were grouped. *Brachygalaxias bullocki* was placed as the sister taxon to *Galaxiella*, these species constituting a basal clade of the Galaxiinae. Within *Galaxias*, *G. truttaceus* and *G. auratus* were grouped, as were *G. fasciatus* and *G. argenteus*. In addition, isolated populations of *G. zebratus* and *G. maculatus* were grouped. However, according to the ML topology, the genus *Galaxias* is polyphyletic. *Galaxias parvus* and *G. olidus* were united as a sister clade to *Paragalaxias*. *Galaxias maculatus* and the Tasmanian mudfish *G. cleaveri* were grouped with *Neochanna*. *Nesogalaxias* was placed in a clade which also comprised *G. paucispondylus* and *G. brevipinnis*-like species (*G. brevipinnis*, *G. vulgaris*, *G. fontanus* and *G. johnstoni*). The lengths of two of the shortest branches were not significantly positive (Fig. 2.6) indicating that those groupings were not well supported.

Maximum parsimony analysis recovered two equally mp trees 917 steps in length (Fig. 2.7). The two mp trees were identical except for the placement of basal members of the [*Nesogalaxias*, *G. paucispondylus*, *G. brevipinnis*, *G. vulgaris*, *G. fontanus* and *G.*

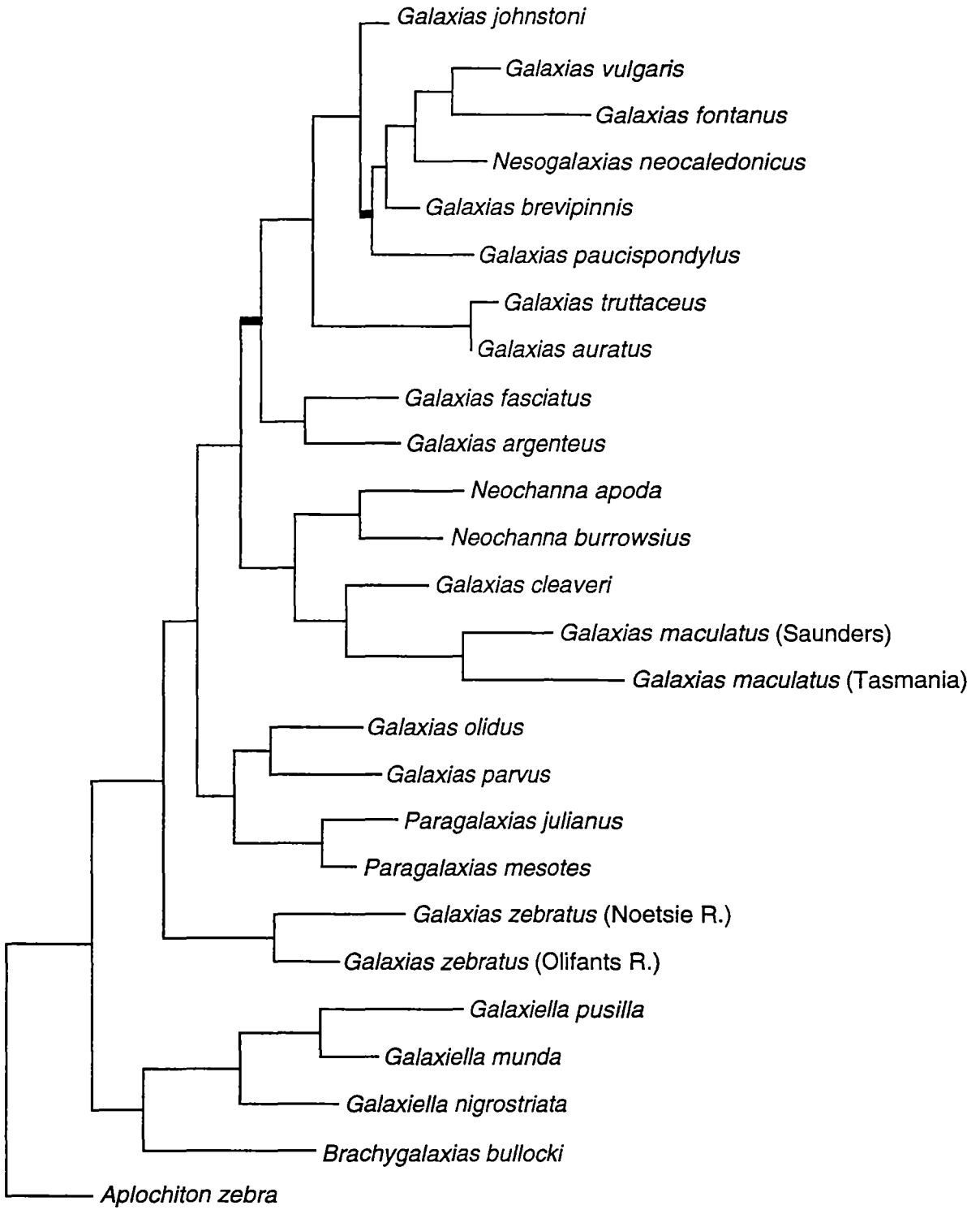

Figure 2.6 Maximum likelihood tree based on 303 bp of cytochrome *b* sequence. The analysis included 25 galaxiine sequences and was rooted with *Aplochiton zebra* as an outgroup The analysis used an expected TV/TI ratio of 2:1. The log likelihood of the tree is -3654.2. Branch lengths that are not significantly positive (0.01) are represented by thickened lines. Branch lengths are proportional to the expected number of nucleotide substitutions.

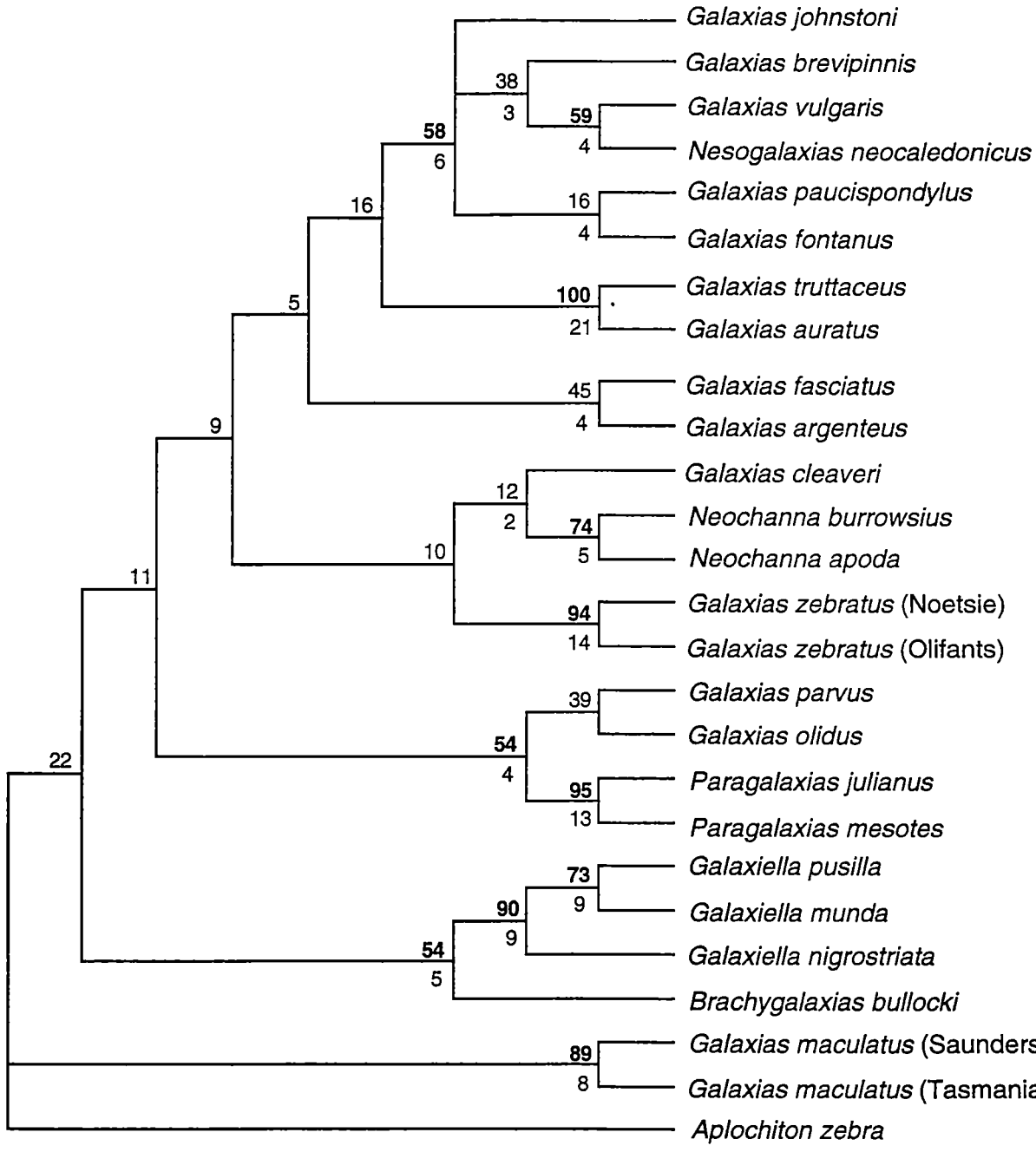

Figure 2.7 Strict consensus of two most parsimonious trees (917 steps) based on 303 bp of cytochrome *b* sequence. The analysis included 25 galaxiine sequences and was rooted with the outgroup *Aplochiton zebra*. TVs were given a weighting of 2. Values at branch points indicate bootstrap support (500 replicates) with values over 50% in bold. Numbers below branch points are Bremer support values >1.

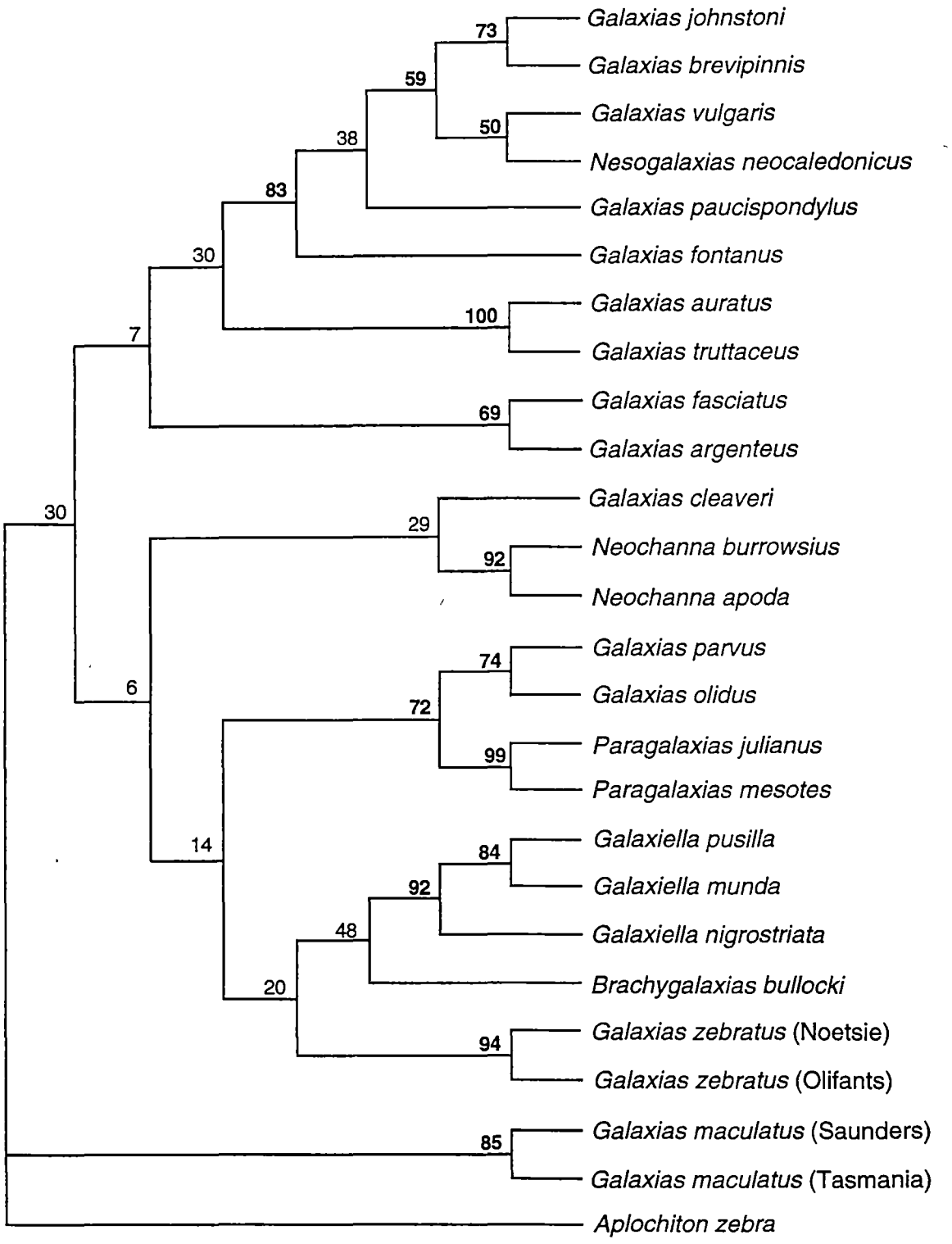

Figure 2.8 Neighbor-Joining bootstrap tree based on 303 bp of cytochrome *b* sequence. The analysis included 25 galaxiine sequences and was rooted with the outgroup *Aplochiton zebra*. Distances were calculated with the maximum likelihood algorithm of Felsenstein (1993) using an expected TV/TI ratio of 2:1. Values at branch points indicate bootstrap support (500 replicates) with values ≥ 50% shown in bold.

G. johnstoni] clade. The mp topology was largely similar to the ML tree, the main differences between them involving the placement of *G. maculatus* and *G. zebratus*. Bootstrapping and Bremer values provided moderate to high support (>50%; >4 steps) for the large *G. brevipinnis* clade and also for [*G. truttaceus*, *G. auratus*], [*Paragalaxias*, [*G. parvus*, *G. olidus*]] and [*Brachygalaxias*, [*G. nigrostriata*, [*G. pusilla*, *G. munda*]]]. As in the ML tree, *G. fasciatus* and *G. argenteus* were grouped, as were *G. parvus* and *G. olidus*, and *G. cleaveri* was closely related to *Neochanna*. However, these three groupings received relatively low support, with bootstrap estimates below 50% and Bremer support values not exceeding four steps. The EOR weighting strategy (weights of 2 for a G-C substitution, 3 for a G-T substitution) had little effect on levels of bootstrap support. Similarly, alternative TI/TV ratios produced some topological changes involving deep splits within the group but had little effect on strongly supported clades.

The neighbor joining (NJ) bootstrap tree (Fig. 2.8) is largely similar to the mp and ML trees. The clades [*G. truttaceus*, *G. auratus*], [*G. fasciatus*, *G. argenteus*], [*Nesogalaxias*, *G. paucispondylus*, *G. brevipinnis*, *G. vulgaris*, *G. fontanus* and *G. johnstoni*], [*Paragalaxias*, [*G. parvus*, *G. olidus*]] and [*Brachygalaxias*, [*G. nigrostriata*, [*G. pusilla*, *G. munda*]]] were supported by moderate to high bootstrap values. In addition, the monophyly of *Galaxiella*, *Paragalaxias*, *Neochanna*, and populations of *G. maculatus* and *G. zebratus* was supported with high bootstrap estimates.

The conservative results of the cytochrome *b* phylogenetic analysis are summarised in Fig. 2.9. Groups that were supported by each analysis method and received substantial bootstrap support are represented by unbroken lines. Conflict between the ML, mp and NJ trees involves groupings that received low levels of bootstrap and Bremer support. For example, in NJ and mp, but not ML topologies, *G. maculatus* was placed as a basal galaxiine (bootstraps 22%, 30%). Distance analysis grouped *G. zebratus* with the [*Brachygalaxias*, *Galaxiella*] clade (20%), but this was not supported by the ML and mp analyses. The arrangement of taxa within the clade [*Nesogalaxias*, *G. paucispondylus*, *G. brevipinnis*, *G. vulgaris*, *G. fontanus*, *G. johnstoni*] was inconsistent. While NJ and mp analyses both united *Nesogalaxias* and G. *vulgaris* with bootstrap estimates of 59% and 50% respectively, this clade was not supported by the ML tree. In addition, [*G. brevipinnis, G. johnstoni*] was strongly supported (73%) by the distance analysis but absent from the mp and ML trees.

When topological constraints were enforced in the mp analysis to make *Galaxias* a monophyletic genus, an additional 86 steps was forced upon the cytochrome *b* data.

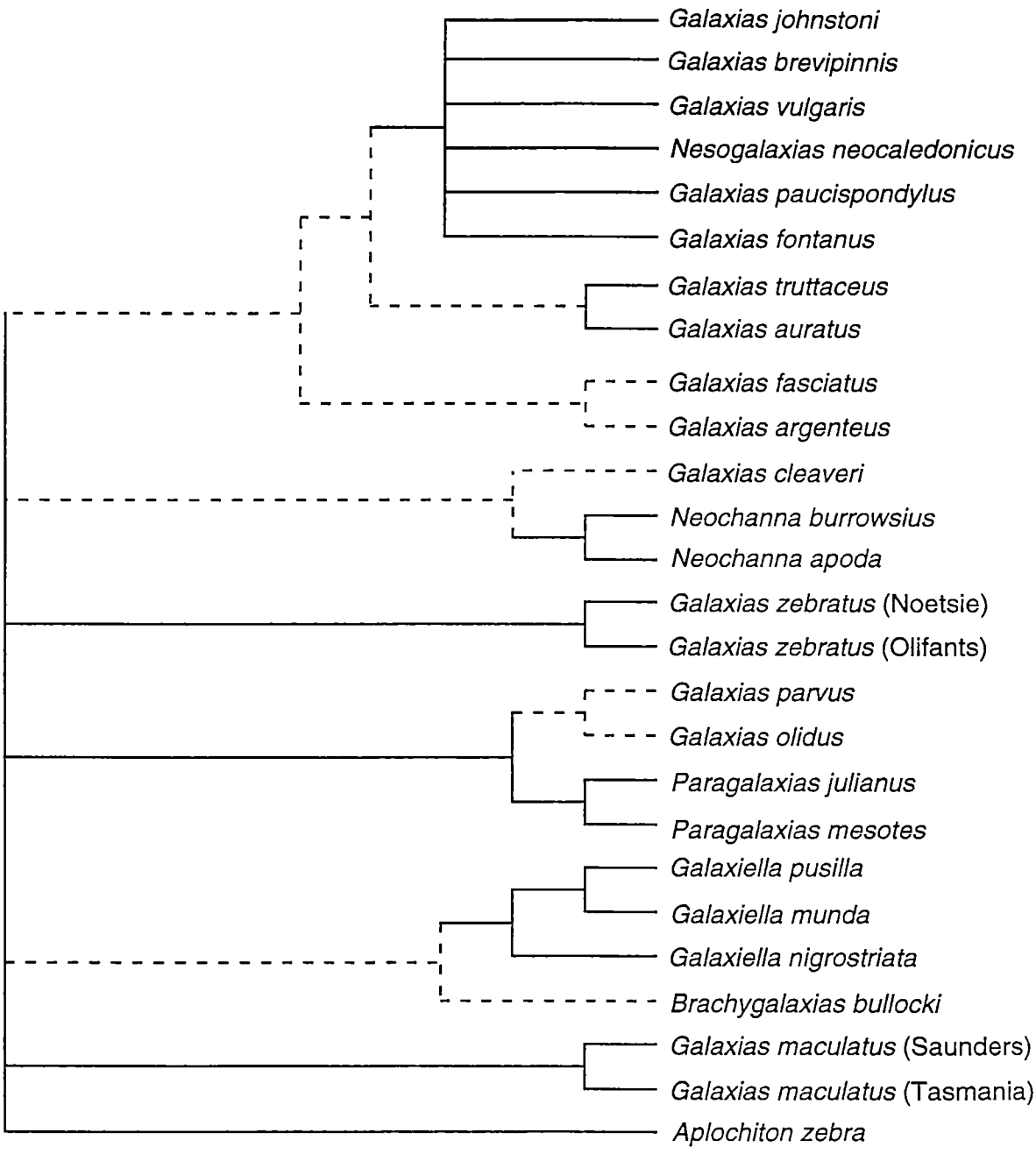

Figure 2.9 A conservative galaxiine cytochrome *b* phylogeny, showing only the groupings common to the maximum likelihood, maximum parsimony and and neighbor-joining trees. Clades that received low levels of bootstrap support are indicated by dotted lines.

The common ancestry of the clades [*G. truttaceus*, *G. auratus*] and [*G. fasciatus*, *G. argenteus*] required 10 extra steps.

Galaxioid cytochrome b phylogeny

Genetic divergences between galaxioid taxa were calculated (Table 2.7) with *Galaxias brevipinnis* as a representative of the Galaxiinae. Pairwise divergences ranged from 23.5% (*G. brevipinnis-Aplochiton*) to 40.1% (*Lepidogalaxias-Lovettia*). *Lepidogalaxias salamandroides* was the most divergent galaxioid sequence, differing from other sequences by a mean of 37.1%. A mean divergence of 28.1% was found between the other four galaxioid cytochrome *b* sequences. Of the observed substitutions, 59% were TIs and 41% were TVs, representing a TI bias of 1.4.

Table 2.7 Percentage sequence divergences (above diagonal) and observed nucleotide substitutions (below diagonal) between galaxioid cytochrome *b* sequences. Numbers in brackets are observed transversion substitutions. Divergence values were calculated with the maximum likelihood algorithm of Felsenstein (1993).

Taxon	*Galaxias*	*Aplochiton*	*Lovettia*	*Lepidogalaxias*	*Retropinna*
Galaxias brevipinnis	-	23.5	27.8	34.5	29.1
Aplochiton zebra	61 (11)	-	23.9	39.5	30.8
Lovettia sealii	66 (25)	59 (21)	-	40.1	33.3
Lepidogalaxias salamandroides	71 (30)	79 (36)	77 (36)	-	34.1
Retropinna tasmanica	66 (30)	70 (31)	72 (32)	71 (30)	-

For parsimony analysis of the galaxioid sequences, the galaxiines were represented by *G. brevipinnis*, *G. argenteus*, and *Nesogalaxias neocaledonicus*. In addition, a number of outgroups were included. These included cytochrome *b* sequences for *Salmo trutta* (EMBL, X77559) and *Oncorhynchus mykiss* (GenBank, L29771) and osmerid sequences from Taylor and Dodson (1994).

Random treelength distribution was examined to assess the phylogenetic information content of the galaxioid cytochrome *b* data. Seven sequences, representing salmonid, osmerid and five galaxioid lineages were included. The length distribution of 10,000 random trees revealed no significant skew to the left ($g1 = 0.04$, $P>0.05$) indicating the absence of strong phylogenetic signal in the cytochrome *b* data set (Hillis and Huelsenbeck 1992). However, when only TVs were analysed, the tree-length distribution was significantly skewed ($g1 = -0.66$, $P<0.01$). Thus, while the data are saturated with TIs, TVs may be phylogenetically informative.

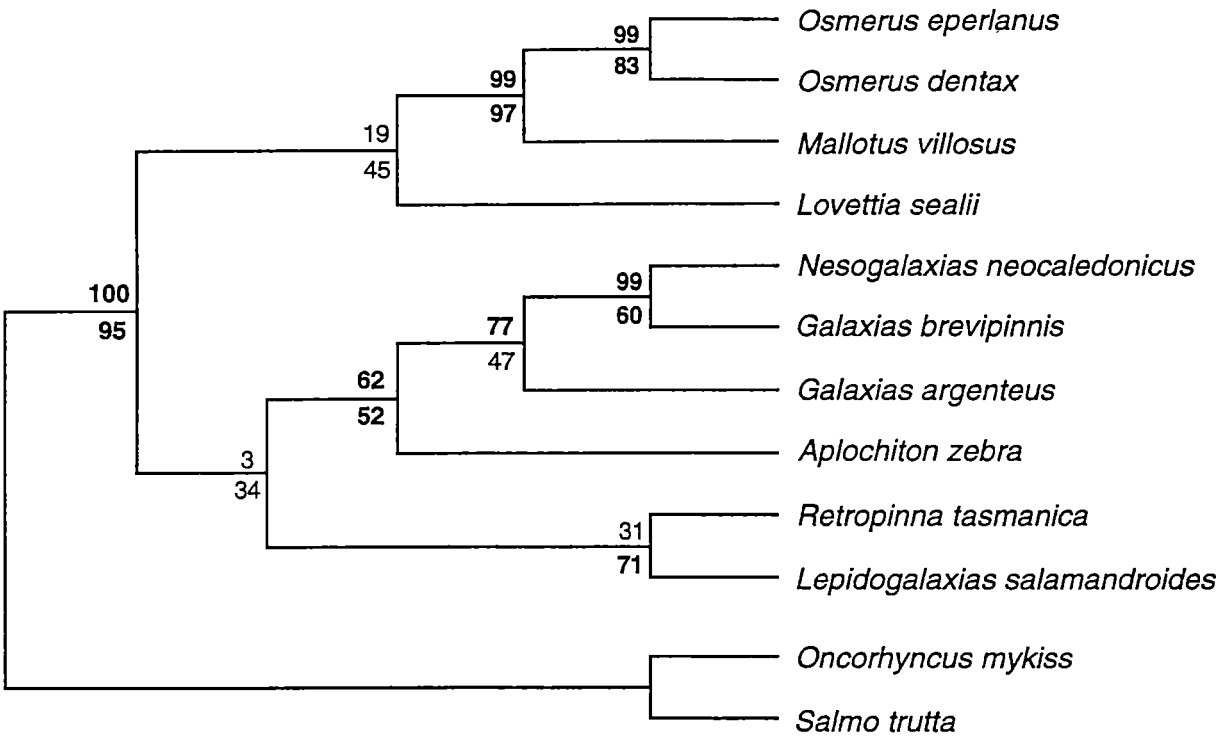

Figure 2.10 Parsimony bootstrap tree for osmeroid cytochrome *b* sequences. Two salmonid taxa were included as outgroups. Bootstrap values (500 replicates) above nodes are for analysis with a TV:TI weight of 2:1, while those below nodes are for analysis of TVs only. Estimates ≥ 50% are shown in bold.

Parsimony analysis was performed with a TV/TI weight of 2:1, and also with TIs excluded (Fig 2.10). The monophyly of the salmonids, osmerids and galaxiines was supported with moderate to high bootstrap values. *Aplochiton* and the galaxiines were grouped, with bootstraps over 50% for both weighting strategies. In contrast, most other groupings received low levels of bootstrap support. *Lepidogalaxias* and *Retropinna* were grouped by TV analysis (71%), but this grouping was only weakly supported (31%) by the alternative weighting strategy. *Lovettia* formed a weakly supported clade with the osmerids (45% and 19%).

The bootstrap support for alternative topologies was examined. *Lovettia* was included with *Aplochiton* and the galaxiines by 9% (2:1) and 4% (TV) of the bootstrap replicates. *Lovettia* was placed as the sister taxon to *Lepidogalaxias* with values of 2% (2:1) and 1% (TV). *Lepidogalaxias* was placed as basal to the osmeroids with 43% (2:1) and 25% (TV) bootstrap support.

2.3.2 16S rRNA gene phylogeny

Up to 521 base pairs of mitochondrial 16S rRNA gene sequence were obtained from members of the Galaxiinae. CLUSTAL produced an alignment 522 base pairs in length (Table 2.8). An alternative 530 bp alignment was obtained with lower gap penalties. The attempt to estimate a secondary structure for the galaxiine mitochondrial 16S rRNA gene was only partly successful. The most conserved regions of the galaxiine sequence fragment were easily folded around the putative secondary structure for cow 16S (Gutell *et al.* 1993). These regions included approximately 200 bp of sequence from the 5' end, and about 140 bp from the 3' end of the fragment. However, the remaining portion (about 180 bp) was too variable to be confidently aligned with the cow sequence. Moreover, the portions of the molecule for which a secondary structure could be confidently estimated contained relatively few of the phylogenetically informative sites.

Intraspecific variation and phylogeography

Mitochondrial 16S rRNA partial gene sequences were obtained from more than one individual of some taxa. Identical sequences were obtained for two *G. truttaceus* from Allens Creek in southeast Tasmania. In contrast, this gene detected considerable divergence between isolated populations of *G. zebratus* and *G. maculatus*.

Galaxias zebratus populations

Two 16S rRNA sequences, representing the Olifants River and Noetsie River populations of *G. zebratus*, were separated by 26 observed substitutions and 5.3% corrected sequence divergence. Of the 26 substitutions, 13 were transitions and 13 were transversions. In addition, two insertion/deletion events were detected between the sequences.

Galaxias maculatus populations

A substantial level of intraspecific diversity was detected between *G. maculatus* isolates. Variation was detected at a total of 30 nucleotide sites. Pairwise sequence divergences ranged from 1.4% (seven substitutions) between the West Falkland and Saunders Island sequences to 5.6% (28 observed substitutions) between the Tasmanian and West Falkland sequences (Table 2.9). The Tasmanian sequence differed from the Falkland Islands sequences by a mean of 5.1%. Sequence divergence values were similar when calculated with the Jukes-Cantor algorithm. Within *G. maculatus*, a single inferred deletion event was inferred in the Tasmanian sequence. Of the observed pairwise substitutions, 62% were TIs while 38% were TVs, representing an observed TI bias of less than 2:1.

Table 2.8 Partial sequences of the mitochondrial 16S rRNA gene (L-strand) of 27 galaxiids. Dots indicate matches with the *Galaxias truttaceus* sequence. Sequence alignment was performed with CLUSTAL.

Galaxias truttaceus	CCCGCCTGCC	CTGTGACTAT	ATGTTTAACG	GCCGCGGTAT	TTTGACCGTG	CAAAGGTAGC
Galaxias auratus	. .. . .	. . .	..			.
Galaxias maculatus (Tasmania)	T . ..	.	GA .	. .	.	.
Galaxias maculatus (WFalkland)	T.	. .	.	. .	. . .	.. .
Galaxias maculatus (Saunders)	.T	. . .	. . .	. .	. .	.
Galaxias brevipinnis	. ... .	... ..	.A .	. .		. .
Galaxias vulgaris	. . . .	. .. .	A .	. .	.	.
Galaxias johnstoni	. .. ..	....	.A . .	. ...	.	
Galaxias fontanus	. .. . .	.	A . ..	...	. . .	
Galaxias paucispondylus	.. . ..	.	A .. .	.	. .	
Galaxias fasciatus	??????????	? ? .	GA			
Galaxias argenteus	.		.A ..	.	.	.
Galaxias parvus	. T.. .	.	. . .	.		.
Galaxias olidus	. ..	. .				
Galaxias zebratus (Olifants)	..T . .	. .	.A .	.	.	
Galaxias zebratus (Noetsie)	..T .	. . .	A	.	T .C	...
Galaxias cleaveri	. T.	. .	. .	. .	.	. .
Neochanna apoda	. .. .	. ....	. .			
Neochanna burrowsius	.. . . ..	. . .	. .	.	.. .	..
Paragalaxias mesotes	...	.	.C..	. .		.
Paragalaxias julianus	. .. .	. ..	.C .	..	..	
Galaxiella munda	. .	. ...	.A .		.	.
Galaxiella nigrostriata	.	. .G	. . ..	. .	. G ..	
Galaxiella pusilla	T.	. C	A . . .	.	. .	
Brachygalaxias bullocki	. .	.	. .	..	. ..	.
Nesogalaxias neocaledonicus	. . .	..	. .	.		
Aplochiton zebra	. T. .. .	. . ...	— ...	..	.. .	

Galaxias truttaceus	GCAATCACTT	GTCTTTTAAA	TGGAGACCTG	TATGAATGGC	AAGACGAGGG	CTAAGCTGTC
Galaxias auratus	. . .	.. .	. . .	. .	.. . .	. ..
Galaxias maculatus (Tasmania)	. . .		A ..C.	— .		
Galaxias maculatus (WFalkland)	. . .	.. .	. A A C.	.	. .	
Galaxias maculatus (Saunders)	....	. .	. A .C	. . .	..	.
Galaxias brevipinnis	....		. .	. ..	.. .	.
Galaxias vulgaris	... .	.... .	.	. .	.	. .
Galaxias johnstoni	.....	. .. ..	.	..	. .	.
Galaxias fontanus	....	... . .	.. . ..		. .	. .
Galaxias paucispondylus	.. .. .	. . . .	. . . .		.. . ..	.. . .
Galaxias fasciatus	.. ..	.C .	. ..	. .		. .
Galaxias argenteus	. .. .	.	. ..	. .	. .	..
Galaxias parvus	... .	. C ..	. . .	... ...	. . . ..	. .
Galaxias olidus	.. . .	. .	. . .	. .	. .	.
Galaxias zebratus (Olifants)	.. .. .	. .	. A ..	. . ..	..	. T
Galaxias zebratus (Noetsie)	... . .	. .. .	. A .	. .. ..		T
Galaxias cleaveri	. ..	.	.	. .	.. .. .	..
Neochanna apoda	. .. .	. .	. .	..	. .	G. .
Neochanna burrowsius	. .... .	.	.	..	. .	.
Paragalaxias mesotes	. .		A	. .	. . .	..
Paragalaxias julianus	. .	. . . ..	..A .	. .	.	.
Galaxiella munda	. . .	...	.A .		.	GA
Galaxiella nigrostriata	.	..	A .	.	TA	C GA
Galaxiella pusilla	.	.	A	. .		GA
Brachygalaxias bullocki	. . .		A C	.	TA	C A
Nesogalaxias neocaledonicus	. ..	. .	. A			. .
Aplochiton zebra	. .	.	A .	.	.	..

Table 2.8-continued.

Galaxias truttaceus	TCCTTTTCCA	AGTCAGTGAA	ATTGATCTCC	CCGTGCAGAA	GCGGGGATAT	TCTCATAAGA
Galaxias auratus	C .	.. .		.. .	. .	. .
Galaxias maculatus (Tasmania)	C .		. ..	. .	.. . TC	C . .
Galaxias maculatus (WFalkland)	. C .	.	.	.. .	. . CA	C C ..
Galaxias maculatus (Saunders)	C .	.	.	.	.CA	C C . .
Galaxias brevipinnis	. . . .	. .	G ..	.	. . .	A .
Galaxias vulgaris	. . . .	. .	G	. . . ..	... . GA	A . ..
Galaxias johnstoni		. .	G	.	. .	A . ..
Galaxias fontanus	. .		. ..	. ...	. C .	AA .
Galaxias paucispondylus	.	. ...	...	. T	.. GC	CA .
Galaxias fasciatus	.	G ..	G . C	. . .	C	CA . .
Galaxias argenteus	..	.	G . .	...		AC
Galaxias parvus	.	. ... .		. .	.	.
Galaxias olidus	.	. .	. . ..	.	.	. ...
Galaxias zebratus (Olifants)	. .	.	. TT .	.	. AA . .	AC
Galaxias zebratus (Noetsie)	.	.	. TT	..	. AA T	A .
Galaxias cleaveri	.	.. . ..		. .	.TA	AAA ..
Neochanna apoda	.. .	.,. .	. .	.. ..	..G	C .
Neochanna burrowsius	. .	..		. ..	.. . GC	CA .
Paragalaxias mesotes	.	. .		.	.. . .	A . .
Paragalaxias julianus	.	. . ..		. .	. .	A .. .
Galaxiella munda	. ..	.. A		.. ..	... ..A	GAA .. .
Galaxiella nigrostriata	C .T	. A .		.. .. .	C	G C .
Galaxiella pusilla	. . C	A . . .		.	.... . .A	AA
Brachygalaxias bullocki	.. AC	. . ..	T	.	A .TA	AA . . ...
Nesogalaxias neocaledonicus	. . .	.	.	. . . .	C	CA
Aplochiton zebra	. C C .	. .		. ...	. ...A	AA ..

Galaxias truttaceus	GCAGAAGACC	CTATGGAGCT	TTAGACACAA	GGCAGCCCAC	GTTAAACAGC	CTCTCTTAAG
Galaxias auratus	CG .	....	. . G	. ....	. .	. . . .
Galaxias maculatus (Tasmania)	CG . . .	.	....G T	A A .T	.A	.C TG GC .
Galaxias maculatus (WFalkland)	CG	.....	. G T	.A A ..T	.. ...	C C GC
Galaxias maculatus (Saunders)	CG .. .	.	G T	A A...T	.	.C .C GC .
Galaxias brevipinnis	CG		.. ..T.	AA . ..T	.. .A	. T G .
Galaxias vulgaris	CG......	. .	. T	AA . .T	... T.A.	.T.G. A
Galaxias johnstoni	CG	.	......T	AA A. .T	.. .A	.. T G .
Galaxias fontanus	CG		.......T	.AA . .T	. . A	.C T G C
Galaxias paucispondylus	CG ...	... .	. . . T	AA. .T	.... .A.	T.A.
Galaxias fasciatus	CG . .	. . .	....T	AA ....T	. C. ....A	C T. .
Galaxias argenteus	CG..... .	.	.T	AA ..T	. . ..A.	. T. .
Galaxias parvus	CG	.... . .	T.	AA. ...T	... ..A.	ACA . A
Galaxias olidus	CG .. .	. ..	.. T	A. .. .T	. . . A	A . A
Galaxias zebratus (Olifants)	CG	. .	A .TG	AA ..	. . ..A	.C A . A
Galaxias zebratus (Noetsie)	CG . .	. . . . .	. A ..TG	AA.. T	. A . .A	C A .
Galaxias cleaveri	CG .	... . ..	.T	AAT. .T	C .G A	CTAAG.
Neochanna apoda	CG . .	... .. .	... .T	AA. . ..	. CTT.. A.	CG.GA . A
Neochanna burrowsius	CG . .	...	..T	A .	CT . A	T AA. A
Paragalaxias mesotes	CG .	... ..	.T	A .. ..T	C . ..	.T.C .
Paragalaxias julianus	CG . .	. .. ..	... ..T	A. . ..T	. C. ..	.T C .
Galaxiella munda	CG . .	. . ....	. ..T.	AA .... T	..C. A.	TC—.A G. A
Galaxiella nigrostriata	CG .. .	.	. . . T	AA T....	.. C A.	TC— A A. A
Galaxiella pusilla	CG .	....	. T	.AA.. .. .	..CC A.	T.TCTA . A
Brachygalaxias bullocki	CG . .	. .	. . T	AAT.. T	.C T.GT AG	.CG A C
Nesogalaxias neocaledonicus	CG	.	. . T.	AA. .T	A	.. G
Aplochiton zebra	CG .		. . T	A. . .	GA AG	— CC C

Table 2.8-continued.

Galaxias truttaceus	GGTATAAACT	TAGTGGC–GC	CTCGCCCAAT	TGTCTTCGGT	TGGGGCGACC	GCGGGGGAAA
Galaxias auratus	. . . .	–A.	.		. .	.
Galaxias maculatus (Tasmania)	. AGAG.	. . –A.	AT TTG C	. .	. .	
Galaxias maculatus (WFalkland)	. AG G	A G–AA	AT TG C	. .	. .	.
Galaxias maculatus (Saunders)	. . GG G .	G–AA	. AT TG C	.	. . .	. . . .
Galaxias brevipinnis	. . AG.	A . . –A	. AT T	. . .	. . .	. . . .
Galaxias vulgaris	G .	A –A.	. AT T.	. .	. . . . .	. .
Galaxias johnstoni	. . . A .	. . A . . –A.	AT T	.	.	.
Galaxias fontanus	. A .	. A . –A.	. AT . T.	.	. .	. . .
Galaxias paucispondylus	A . GAG .	. . A . . –A	. AT . T T	. . .	.	. .
Galaxias fasciatus	C A .	A –AA	AT T . T	.	.	. . .
Galaxias argenteus	A . .	A . . . –A	. AT T	. . . .	. .	.
Galaxias parvus	. . AG	. . –A	TTT			.
Galaxias olidus	. . . AG	–A.	. TTT	. .	. .	. .
Galaxias zebratus (Olifants)	A A A . .	TAT	. T TTT	. T	.	. . T.
Galaxias zebratus (Noetsie)	. . C. . .	A T–TT	. T . TTT	T .	. .	. T.
Galaxias cleaveri	C . AG.	. A . –	T TTT	.	. . . . .	. . T.
Neochanna apoda	. C . G . .	A . T–	T TTT	. . .	. .	. . . C
Neochanna burrowsius	A . CGAG. .	. . . . –	T TTT	. .	.	. . T.
Paragalaxias mesotes	A AG. .	. . A –C	AT T .	. .	.	.
Paragalaxias julianus	A . . AG . .	A . –C	AT T	. .	. .	.
Galaxiella munda	AAGGA .	. A. T–A	. TAT T C	. . .	. .	. . T
Galaxiella nigrostriata	A A . A	. –A	TAT T C	. .	. . T .	. . . C
Galaxiella pusilla	AAG A .	. . –A	TAT T C	. . .	. . . .	. T.
Brachygalaxias bullocki	A AG G . C	CGT –A	. TATTT C	.	.	T . A . T
Nesogalaxias neocaledonicus	. . . AG . .	A. . –A.	. AT T . T	. .	. .	.
Aplochiton zebra	A	. GC. . –AA	GATTT C	. .	. . .	.

Galaxias truttaceus	GACTAGCCCC	CATGTGGAAT	GGGGGTACAT	ACTCCCTGCA	GCCGAGAAAA	ACTTTTCTAA
Galaxias auratus	. . .	. . . .	. . .	. . .	. . . .	. . . . . .
Galaxias maculatus (Tasmania)	AGAC. . .	 C	. A G A .	. –T . AG	. . TA . GTT	. CAC . . .
Galaxias maculatus (WFalkland)	AA. .	 CC	AA. AT	–T AG	. T GGTT	C C
Galaxias maculatus (Saunders)	AGAA. . .	 CC	AA . AT	. –T. AG	. TA . GGTT	C C. . .
Galaxias brevipinnis	C.	. . G.	. A T	TT. –. CAG	. . . . GG	C. . . .
Galaxias vulgaris	. . C. . .	. . .	. A . T	TT–– CAG	. . . TG	C. . .
Galaxias johnstoni	. . C. . . .	. . . . . .	. A . T	TT –. CAG	. . G	. C . . .
Galaxias fontanus	. . C. . .	. . . . .	. A . T .	CT T. . CAG	A . . . G	C . . . .
Galaxias paucispondylus	. . . . .	. . . . .	AA T	TT. –– CAG	. . A. . . GG	. C. . .
Galaxias fasciatus	A C .	. .	AA ––	CT . T. A. .	A. . . G.	. . .
Galaxias argenteus	A. C	. . . .	. TT–––	GT . T . A	. A . . T	. . . . .
Galaxias parvus	A C . .	. . G	. A C TGC	T. –T . AA	A . A. . . T	. . .
Galaxias olidus	A . AC .	.	. . A C TGA	T –T. . . AG.	. . . T.	. . . C
Galaxias zebratus (Olifants)	A AG	C . A	. AA . T	–––– . A	. A . G	. . .
Galaxias zebratus (Noetsie)	A G. . . .	. . CA	A TT	C––– A	A . . . G	. .
Galaxias cleaveri	A . C. .	.	A . ––	T. . –. . A .	A A. . .	. C. . .
Neochanna apoda	AG C	. G.	. . A –– A	C –. . . .	. A . G	. C . . . . .
Neochanna burrowsius	C. . . .	. . G	A . –– G	C – .	A . .	. C. .
Paragalaxias mesotes	A . C .	G.	A T	T –T AA	. T . T	. . . .
Paragalaxias julianus	A. C	. . G	A T	T –T . AA.	. . T. T	. . .
Galaxiella munda	A A–. . .		AA– AT	TT. T CAA.	. A . . T	. . . . . .
Galaxiella nigrostriata	A –A . .	. C	. –A– TT	TA T. . AA.	A . A. . . . T	C . .
Galaxiella pusilla	A C	. .	A AA–––TA	TT T AG	A . A . T	. .
Brachygalaxias bullocki	ACAC T	C	–– AA–GGTA	CAC TT. AG	A. . . .	. .
Nesogalaxias neocaledonicus	A C.	.	. AA TG	TT – CAGG	A TG	C .
Aplochiton zebra	A T	. C	. A. ––	. –T A	A CT	CG . .

Table 2.8-continued.

Galaxias truttaceus	GCAACAGAAA	TTCTGACCAA	AAATGATCCG	GCAAGGCCGA	TCAACGGACC	CAGTTACCCT
Galaxias auratus	. . .	.	. . .	.	.	.
Galaxias maculatus (Tasmania)	. . . . .	. . —	GG. . . .	. TA .	. .	. .
Galaxias maculatus (WFalkland)	. .	. —	G. C .	. TA	.	.
Galaxias maculatus (Saunders)	. . . .	. . . . . —	G C . . .	. . TA .	. .	.
Galaxias brevipinnis	. . . . .	.		. A .	.	. . .
Galaxias vulgaris	. . . . . .	. .	.	. . A .	. . .	.
Galaxias johnstoni	. . . . .		. . . .	.		. .
Galaxias fontanus	. . . . . .	. . .	. .	. GA	. .	.
Galaxias paucispondylus	. .	. .		. . A .	. . .	. .
Galaxias fasciatus	. . .		.	. T .		. . .
Galaxias argenteus	. . . .	. .	. .	A		.
Galaxias parvus	T . . .	. . . .	T .	. T .	A .	. .
Galaxias olidus	.	.	T .	. . . T . .	A	.
Galaxias zebratus (Olifants)	. . . . T	. . . .	. .	C . .	. A .	.
Galaxias zebratus (Noetsie)	. .	. . . .	. . .	T . .	. . T	.
Galaxias cleaveri	. . . . .	. . . . .	. .	. . T . .		. . .
Neochanna apoda	. . . .	. . .	— . . .	. GT .	. .	. . .
Neochanna burrowsius	. .	.	. . C	T . .	. .	
Paragalaxias mesotes	. .	. . .	T	T . . .	.	. . .
Paragalaxias julianus	. . . .		. T . .	T .	. .	
Galaxiella munda	. . . .	. . —	G. . G.	A . .	. . . . . .	. .
Galaxiella nigrostriata	. . . T	. . . . —.	. . .	. GA . . .	. . .	. . . .
Galaxiella pusilla	. . . .	. —.	. .	. A . .	.	. . .
Brachygalaxias bullocki	. C	A T .	T . A . . .	. . T .	. .	. .
Nesogalaxias neocaledonicus	. . .		. .	. GA .	. . .	. .
Aplochiton zebra	. .	. . .	. .	C . . .	. .	. .

Galaxias truttaceus	AGGGATAACA	GCGCAATCCT	CTCCCAGAGT	CCCTATCGAC	GAGTGGGTTT	ACGACCTCGA
Galaxias auratus	. . .	. . . .	. . . .	. . . .		. . . .
Galaxias maculatus (Tasmania)	. . . .	. C	. . . . .	. . . .	. G . . .	. .
Galaxias maculatus (WFalkland)	. . . . .	. . . C	. . A	. . .	G .	. . . . .
Galaxias maculatus (Saunders)	. . . . .	 C	. . . . .	. . .	G	. . . . .
Galaxias brevipinnis	. . . . . .	. . . .	. . .	. . . .	G.	. . . . .
Galaxias vulgaris	. . . . .	G. .	. . . .	. . . . . .	G . .	. . . .
Galaxias johnstoni	. . . . . .	. . . .	. . . . . .	. . . . . .	. G . . .	. . . . .
Galaxias fontanus	. . . .	. .	. . . .	. . . .	G . . .	. . . .
Galaxias paucispondylus	. . . . . .	. .	. . . .	. . . .	G	. . .
Galaxias fasciatus	. . . . . .	. .	. .	. . .	G. . .	. . . . . .
Galaxias argenteus	. . . .	. . . .	. . . . . .	. . . . . .	G.	. . .
Galaxias parvus	. . . .		. . . .	. . .	. G . .	. . . . .
Galaxias olidus	. .	.	. . . .	. . .	G . . .	. . . .
Galaxias zebratus (Olifants)	.	. . . .	T . .	. . . . . .	G.	. . . . .
Galaxias zebratus (Noetsie)	.	. . . .	. . . . .	. . .	. . G .	. . . . . .
Galaxias cleaveri	. . . . . .	. .	. . . .	. .	. . G	. . . . .
Neochanna apoda	. . .		. . . . . .	. . .	. G . . .	. . .
Neochanna burrowsius	. .	. .	. . . .	. . . .	. G. . .	. . .
Paragalaxias mesotes	. . . .	. .	. . . .	. . . . . .	. . . G . .	. . .
Paragalaxias julianus	. . . .	. . . .	. . . . .	. .	G .	. . .
Galaxiella munda	.	. . . . .	. .	. . . .	A. . .	. . . .
Galaxiella nigrostriata	.	. . .	.	. .	G .	. . . .
Galaxiella pusilla	.	. . . .	. . .	. .	G .	. . .
Brachygalaxias bullocki		. .	. .	. .	G . .	.
Nesogalaxias neocaledonicus			T	C .	A	. .
Aplochiton zebra	. . . .	C	T	. . . .	G .	.

Table 2.8-continued.

Galaxias truttaceus	TGTTGGATCA	GGACATCCTA	ATGGTGCAGC	CGCTATTAAG	GG
Galaxias auratus	..... .	.. . .	. .	..	.
Galaxias maculatus (Tasmania)	...	. .	. .	. . ..	.
Galaxias maculatus (WFalkland)	. . . .	. .	..	. .. .	
Galaxias maculatus (Saunders)	. . .	. .	. .. .	. . .	
Galaxias brevipinnis	.. .	.. . .	.	. .	
Galaxias vulgaris	. .	. . . .	. .	. ...	
Galaxias johnstoni	. .	..	. .	. . ...	
Galaxias fontanus	.	. .. .	. . .	. ...	.
Galaxias paucispondylus	. .. .	. . .	.. . .	. .	.
Galaxias fasciatus	..	..	. .. .	. .	
Galaxias argenteus	..	. .. .	.. .	.. .	
Galaxias parvus	. ..	.. .	.	. ...	
Galaxias olidus		.....	. .	.	
Galaxias zebratus (Olifants)	. .	.	.. . ..	...	
Galaxias zebratus (Noetsie)	.. .	.. .	. .	.. . .	
Galaxias cleaveri	... .	...	.... .	. G.....	
Neochanna apoda	. ..	..	. ... .	. ...	.
Neochanna burrowsius	. . .	. . .	C. . ..	. . G. ...	
Paragalaxias mesotes	. .	. ..	.	. . ...	.
Paragalaxias julianus	. .	... . . .	.	... ..	.
Galaxiella munda	. .	. .	..	....	.
Galaxiella nigrostriata	. .. .	. . .	. . .. .	. ....	
Galaxiella pusilla	...	.	. . .A.	.... ..	.
Brachygalaxias bullocki	. ..	.... .	T. . — .	... .	.
Nesogalaxias neocaledonicus	. .	. .. .	.. ..	. G.....	.
Aplochiton zebra	. .	.. ..	. .	....	.

Table 2.9 Percentage sequence divergences (above diagonal) and observed nucleotide substitutions (below diagonal) between *Galaxias maculatus* 16S rRNA sequences. Figures in brackets are observed numbers of transversion substitutions. Divergence values were calculated with the maximum likelihood algorithm of Felsenstein (1993).

Taxon	Tasmania	West Falkland	Saunders Island
G. maculatus (Tasmania)	–	5.6	4.6
G. maculatus (West Falkland)	28 (11)	–	1.4
G. maculatus (Saunders Island)	23 (11)	7 (0)	–

Parsimony analysis of the *G. maculatus* 16S rRNA sequences was conducted with the inclusion of *G. brevipinnis* and *G. truttaceus* as outgroups. A topology identical to the cytochrome *b* tree for the same populations (Fig. 2.3) was recovered. The Saunders Island and West Falkland genotypes were grouped with 100% bootstrap support, as were the three *G. maculatus* sequences.

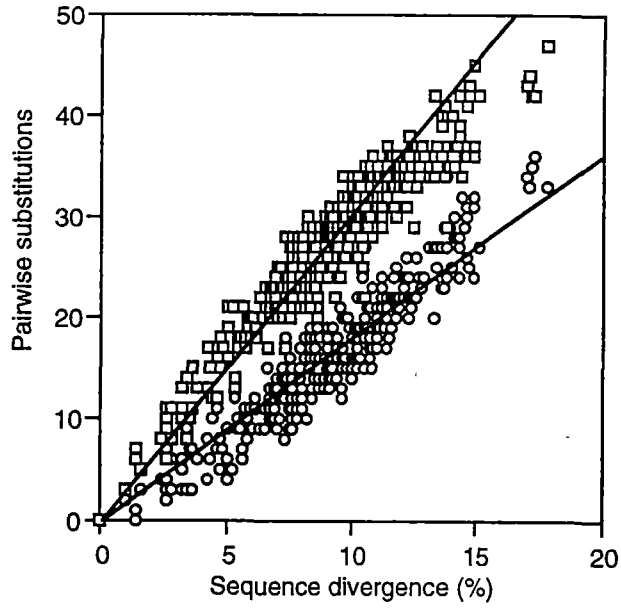

Figure 2.11 Observed TIs (open squares) and TVs (open circles) plotted against pairwise 16S rRNA sequence divergence. Divergence values were calculated with the maximum likelihood algorithm of Felsenstein (1993) and are corrected for multiple hits. Linear regression lines for TIs (r2 = 0.92) and TVs (r2 = 0.89) are shown.

Interspecific variation

As for the cytochrome *b* analysis, the 16S rRNA phylogeny was initially restricted to 25 galaxiine sequences, representing 23 extant species of the Galaxiinae, and the South American *Aplochiton zebra* as an outgroup. *Galaxias zebratus* and *G. maculatus* were each represented by two divergent sequences (Noetsie and Olifants River *G. zebratus*;

Table 2.10 Percentage sequence divergences (above diagonal) and observed number of nucleotide substitutions (below diagonal) for pairwise comparisons of galaxiid 16S rRNA sequences. Divergence values were calculated with the maximum likelihood algorithm of Felsenstein (1993).

Taxon	*Gtr*	*Gau*	*Gmt*	*Gms*	*Gbr*	*Gvu*	*Gjo*	*Gfo*	*Gpc*	*Gfa*	*Gar*	*Gpv*	*Gol*	*Gzo*	*Gzn*	*Gcl*	*Nap*	*Nbu*	*Pme*	*Pju*	*Glm*	*Gln*	*Glp*	*Bbu*	*Nne*	*Aze*
G. truttaceus	–	1.0	12.5	12.0	6.7	7.3	6.1	7.3	8.0	8.4	6.1	8.0	6.7	10.0	9.8	8.4	8.2	7.3	6.9	6.9	11.7	12.6	12.1	17.3	8.4	8.7
G. auratus	5	–	12.2	11 8	6.4	7.1	5.8	7.1	7.7	8.1	5 8	7.7	6.5	9.3	9.3	8.6	8.4	7.5	6.9	6.9	11.4	12.3	11.8	17.0	8.1	8.1
G. maculatus (T)	59	58	–	4.6	10.2	10.7	10.6	10.2	10.0	10.5	9.8	11.4	10.4	13.8	14.3	10.8	12.6	11.7	11.4	11.4	12.3	13.8	13.2	17.1	10.5	11.7
G. maculatus (S)	57	56	28	–	11.2	11.1	11.4	11.2	11.2	12.4	11.4	13.7	12.3	14.7	14.2	12.6	13.9	13.6	12.3	12.3	12.6	14.2	14.9	17.8	11.1	11.9
G. brevipinnis	33	32	49	53	–	1.6	1.4	2.5	2.5	4.7	2.8	6.3	5.4	10.0	9.5	6.9	8.7	7.1	4.2	4.2	7.6	10.4	8.9	14 6	3 5	8.1
G. vulgaris	36	35	51	53	8	–	2.6	3 2	3.2	5.8	3.6	7.1	5.8	10.7	10 2	7.4	9.4	7.6	5.4	5.4	7.8	11.0	9.1	14.4	4.2	7.8
G. johnstoni	30	29	51	54	7	13	–	2.4	3.2	4.3	2.6	7.1	5.8	10.0	9.3	7.4	9.1	8.0	5.0	5.0	7.6	10.0	8.9	14.8	4.2	8.3
G. fontanus	36	35	49	53	13	16	12	–	3.2	4.7	3.4	7.8	6 9	9.4	8.5	6.9	8.5	7.6	6.0	6.0	7.0	9.1	8.5	14.1	3.8	8.3
G. paucispondylus	39	38	48	53	13	16	16	16	–	4.7	4.2	8.0	7.1	10.2	10.0	7.6	10.0	6.7	5.6	5.6	7.0	9 6	8.7	14.9	3.4	8.1
G. fasciatus	40	39	49	57	23	28	21	23	23	–	3.5	7.5	7.5	9.6	8.7	6.7	8.6	7.5	6.0	6.0	8.3	11.0	8.9	14.5	5.5	8.7
G. argenteus	30	29	47	54	14	18	13	17	21	17	–	5.9	5.3	8.3	8.5	6.5	8.1	7.6	4.4	4.4	7.2	9.7	8.3	13.7	4.6	7.4
G. parvus	39	38	54	64	31	35	35	38	39	36	29	–	2.8	9.1	9.8	6.1	7.6	6.9	5.2	5.2	10.3	10.8	9.7	14.1	7.3	9.0
G. olidus	33	32	50	58	27	29	29	34	35	36	26	14	–	9.1	10.2	7.2	7.6	6 9	5.0	5.0	9.6	11.0	9.8	13.3	6.2	8.3
G. zebratus (O)	48	45	64	68	48	51	48	45	49	45	40	44	44	–	5.3	9.1	10.1	9 5	10.2	10.2	9.6	10.8	10.8	14.2	10.7	9.3
G. zebratus (N)	47	45	66	66	46	49	45	41	48	41	41	47	49	26	–	8.1	9.8	9.8	9.5	9.5	10.4	12.9	12.2	13.7	10.5	10.4
G. cleaveri	41	42	51	59	34	36	36	34	37	32	32	30	35	44	39	–	6.7	4 8	6.7	6.7	8.9	11.0	8.9	11.8	7.4	8.1
N. apoda	40	41	59	64	42	45	44	41	48	41	39	37	37	48	47	33	–	5.0	8.4	8.4	11.2	11.5	11.4	13.4	10.2	10.2
N. burrowsius	36	37	55	63	35	37	39	37	33	36	37	34	34	46	47	24	25	–	6.7	6.7	9.6	11.0	9.1	13.6	8.0	9.0
P. mesotes	34	34	54	58	21	27	25	30	28	29	22	26	25	49	46	33	41	33	–	0.0	8.5	10 5	9.2	14.6	5.6	7.8
P. julianus	34	34	54	58	21	27	25	30	28	29	22	26	25	49	46	33	41	33	0	–	8.5	10.5	9.2	14.6	5.6	7.8
G. munda	55	54	58	59	37	38	37	34	34	39	35	49	46	46	49	43	53	46	41	41	–	6.7	5.5	13.6	7.2	9.5
G. nigrostriata	59	58	64	66	50	52	48	44	46	51	46	51	52	51	60	52	54	52	50	50	33	–	7.2	14.6	9.4	10.5
G. pusilla	57	56	62	69	43	44	43	41	42	42	40	46	47	51	57	43	54	44	44	44	27	35	–	15.1	9.1	10.2
B. bullocki	78	77	77	80	67	66	68	65	68	65	63	65	62	65	63	55	62	63	67	67	63	67	69	–	14.6	13.2
N. neocaledonicus	41	40	50	53	18	21	21	19	17	27	23	36	31	51	50	36	49	39	28	28	35	45	44	67	–	8.3
A. zebra	42	39	55	56	39	38	40	40	39	41	36	43	40	44	49	39	48	43	38	38	45	49	48	61	40	–

Tasmanian and Saunders Island *G. maculatus*). Of the 522 nucleotide positions sampled, 366 (70%) were invariant, while 156 (30%) were variable.

Based on all pairwise comparisons of galaxiine 16S rRNA sequences, 65% of the observed substitutions were TIs while 35% were TVs, representing a TI bias of less than 2. When pairwise comparisons were limited to species differing by less than 10% sequence divergence, the proportions of TIs and TVs were 68% and 32% respectively, or a TI bias of just over 2. Observed numbers of TIs and TVs were plotted against corrected sequence divergence for pairwise comparisons (Fig. 2.11). Linear relationships were detected between genetic distance and both TIs and TVs.

For interspecific comparisons, sequence divergences ranged from 0.0% to 17.8% (Table 2.10). A wide range of values was recorded for intrageneric comparisons. Specifically, 5.0% divergence was detected within *Neochanna*, 0.0% within *Paragalaxias*, and a mean of 6.5% between species of *Galaxiella*. The *Nesogalaxias neocaledonicus* 16S rRNA sequence was separated from that of *G. brevipinnis* by only 3.5% divergence. A mean divergence of 7.6% of was detected between members of the genus *Galaxias*, with a minimum of 1.0% (*G. truttaceus* and *G. auratus*) and a maximum of 14.7% (*G. zebratus* and *G. maculatus*). Relatively few pairwise comparisons revealed less than 5% sequence divergence. A mean of 2.3% divergence separated species in the group containing *G. brevipinnis*, *G. vulgaris*, *G. johnstoni* and *G. fontanus*. *Galaxias fasciatus* and *G. argenteus* sequences differed by 3.5% (17 substitutions). The Tasmanian mudfish *G. cleaveri* was separated from New Zealand mudfish genus *Neochanna* by a mean of 5.8% divergence. *Galaxias parvus* and *G. olidus* were separated by 2.8% divergence (14 substitutions). *Galaxias zebratus* (mean divergence from other taxa = 10.4%), *G. maculatus* (11.7%), *B. bullocki* (14.6%) and *Aplochiton zebra* (9.2%) were each separated from all other taxa by a considerable amount of sequence divergence.

Galaxiine phylogeny from 16S rRNA

Initially, an attempt was made to assess the phylogenetic information content of the data. With the inclusion of all galaxiine sequences, the length distribution of 10,000 random trees showed a significant skew to the left ($g1$ = -0.91, P<0.01) indicating a strong phylogenetic signal in the 16S rRNA data (Hillis and Huelsenbeck 1992). When only 10 representatives of the most divergent galaxiine lineages were included in this analysis, the tree-length distribution was considerably less skewed, indicating that sequences representing the deepest phylogenetic splits are nearly randomised with respect to phylogenetic history ($g1$ = -0.16, P=0.05).

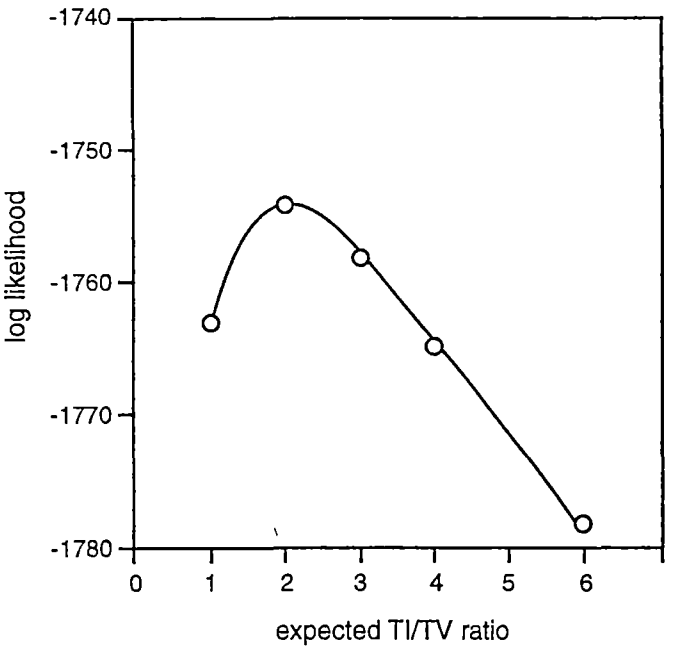

Figure 2.12 Log likelihood of maximum likelihood trees calculated from nine galaxiine16S rRNA sequences under a range of expected TI/TV ratios. The tree with the highest log likelihood (-1754.4) had an expected TI/TV ratio of two.

Again, maximum likelihood (ML) methods were used to determine the optimal TI bias for the 16S rRNA data set. The same subset of nine galaxiine taxa (excluding closely related species) was chosen for ML analysis. The log likelihood of the ML tree was calculated using a range of TI/TV ratios. The optimal tree was recovered with a weighting of 2:1 (Fig 2.12). The log likelihoods of trees produced with TI/TV ratios of 2:1 and 3:1 were considerably greater than those attained with higher and lower ratios, suggesting that they provide a reasonable estimate of the actual TI bias. Because the optimal TI bias also approximated the observed TI bias, a TI/TV ratio of 2:1 was used in subsequent 16S rRNA phylogenetic analyses.

Maximum likelihood analysis of the galaxiine 16S rRNA data recovered a tree with a log likelihood of -3169.8 (Fig 2.13). This tree supported the monophyly of the genera *Neochanna*, *Paragalaxias* and *Galaxiella*. Within *Galaxias*, *G. maculatus* populations were grouped as basal galaxiines. *Galaxias truttaceus* and *G. auratus* were grouped, while *G. fasciatus* and *G. argenteus* were placed as basal to the *G. brevipinnis* clade. According to the ML topology, the genus *Galaxias* is polyphyletic. For example, *Galaxiella* was grouped with members of *Galaxias*, and *Brachygalaxias bullocki* was placed as the sister taxon to *G. zebratus*. *Galaxias parvus* and *G. olidus* were united as a sister group of *Paragalaxias*, and *G. cleaveri* was grouped with *Neochanna*. In addition, *Nesogalaxias* was placed in a clade which also contained *G. paucispondylus*,

G. brevipinnis, *G. vulgaris*, *G. fontanus* and *G. johnstoni*. Analysis of the alternative 530 bp alignment produced a similar ML tree.

Maximum parsimony analysis recovered 17 mp trees 599 steps in length (Fig. 2.14). Areas of conflict between these trees were restricted to the relationships within *Galaxiella*, the *G. brevipinnis* clade, and the arrangement of basal clades. The parsimony trees were generally congruent with the ML topology, and nine groups received substantial levels of bootstrap support, including [*G. cleaveri*, *Neochanna*] and [*G. olidus*, *G. parvus*]. However, unlike the ML tree, the mp topology grouped *Galaxiella* with *Brachygalaxias*, a placement that received low bootstrap and Bremer support (34%, 2 steps). In addition, *G. fasciatus* and *G. argenteus* were united as sisters of the *G. brevipinnis* clade. The EOR weighting strategy (weights of 3 for a G-T substitution, 2 for a G-C substitution) had little effect on levels of bootstrap support. Similarly, analysis of the alternative 530 bp alignment, and alternative TI/TV ratios produced some topological changes involving deep splits within the group but had little effect on strongly supported clades.

The neighbor joining (NJ) bootstrap tree (Fig. 2.15) provided levels of bootstrap support that were generally similar to those in the parsimony analysis. However, a few clades received substantially more support from distance analysis, including the clades [*G. fasciatus*, *G. argenteus*], [*Nesogalaxias*, *G. paucispondylus*] and [*Nesogalaxias*, *G. paucispondylus*, *G. brevipinnis*, *G. vulgaris*, *G. fontanus*, *G. johnstoni*]. Again, most conflict between the NJ and other topologies involved groupings that received low levels of statistical support. A major exception was the clade [*G. fasciatus*, *G. argenteus*] which received 59% support in the NJ bootstrap tree but was not present in the ML tree.

A conservative galaxiine 16S rRNA phylogeny is illustrated in Fig. 2.16. Groups that were supported by each analysis method and received substantial bootstrap and Bremer support are represented by unbroken lines. Consistent but poorly supported groupings are represented by dashed lines.

When topological constraints were enforced to make *Galaxias* a monophyletic genus, an additional 87 steps was forced upon the 16S rRNA data. Furthermore, the common ancestry of the [*G. truttaceus*, *G. auratus*] and [*G. fasciatus*, *G. argenteus*] required an additional 15 steps.

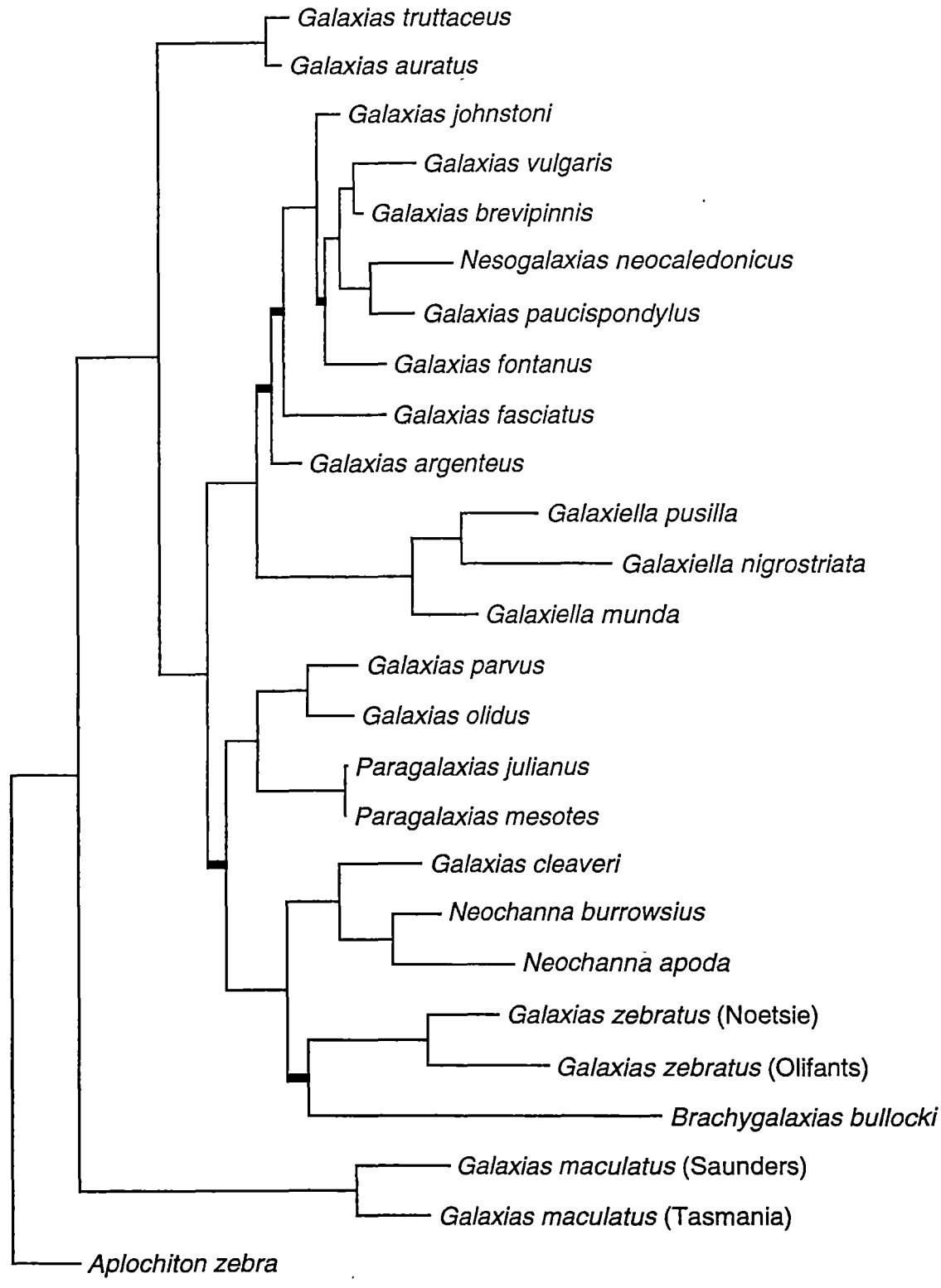

Figure 2.13 Maximum likelihood tree based on 16S rRNA sequences. The analysis included 25 galaxiine sequences and was rooted with *Aplochiton zebra* as an outgroup. The analysis used an expected TI/TV ratio of 2:1. The log likelihood of the tree is -3169.8. Branch lengths that are not significantly positive (0.01) are represented by thickened lines. Branch lengths are proportional to the expected number of nucleotide substitutions.

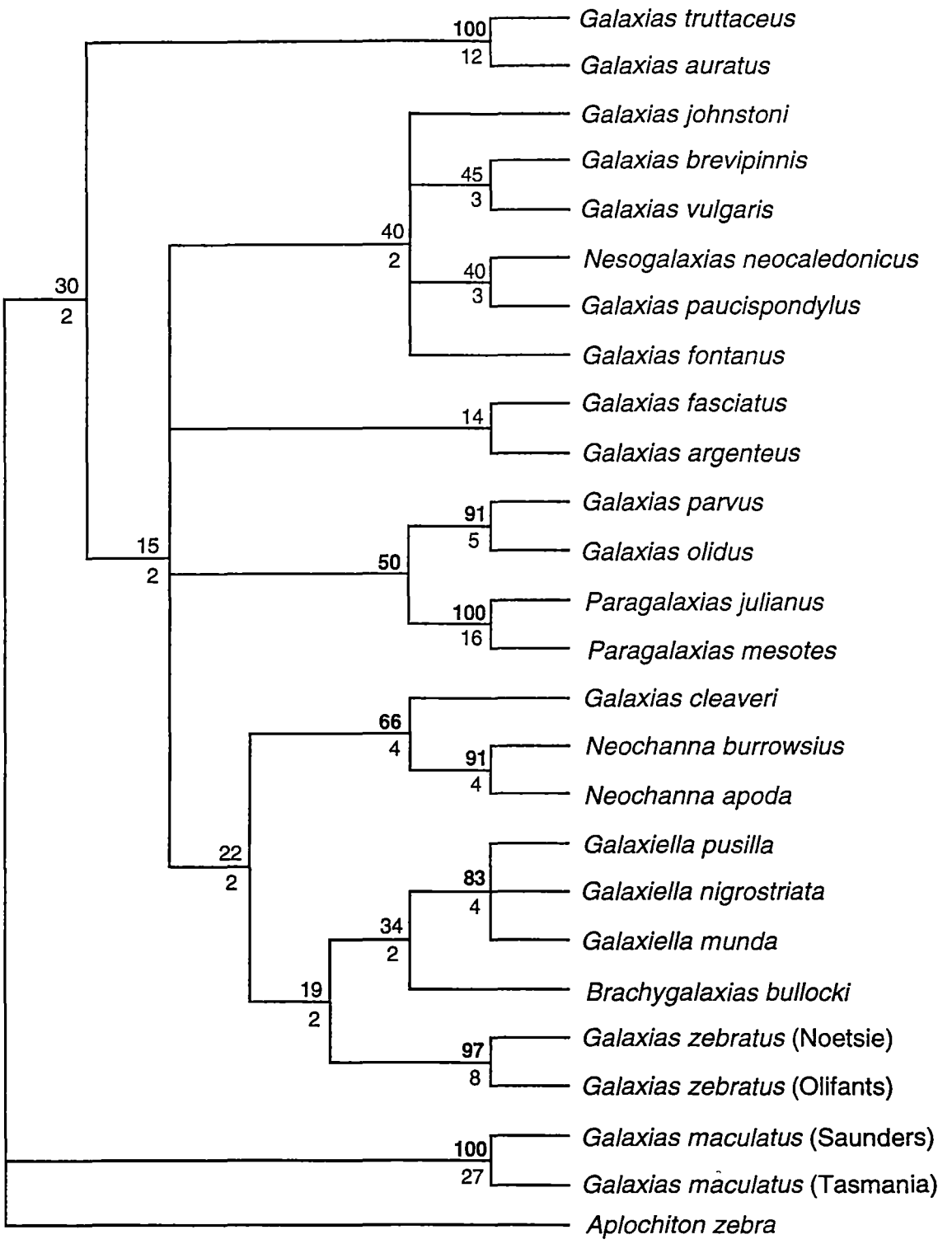

Figure 2.14 Strict consensus of 17 most parsimonious trees (599 steps) from 16S rRNA sequences. The analysis included 25 galaxiine sequences and was rooted with the outgroup *Aplochiton zebra*. Values above branch points indicate bootstrap support, as based on 500 resamplings of the data. Bootstrap estimates ≥ 50% are shown in bold. Numbers below branch points are Bremer support values >1.

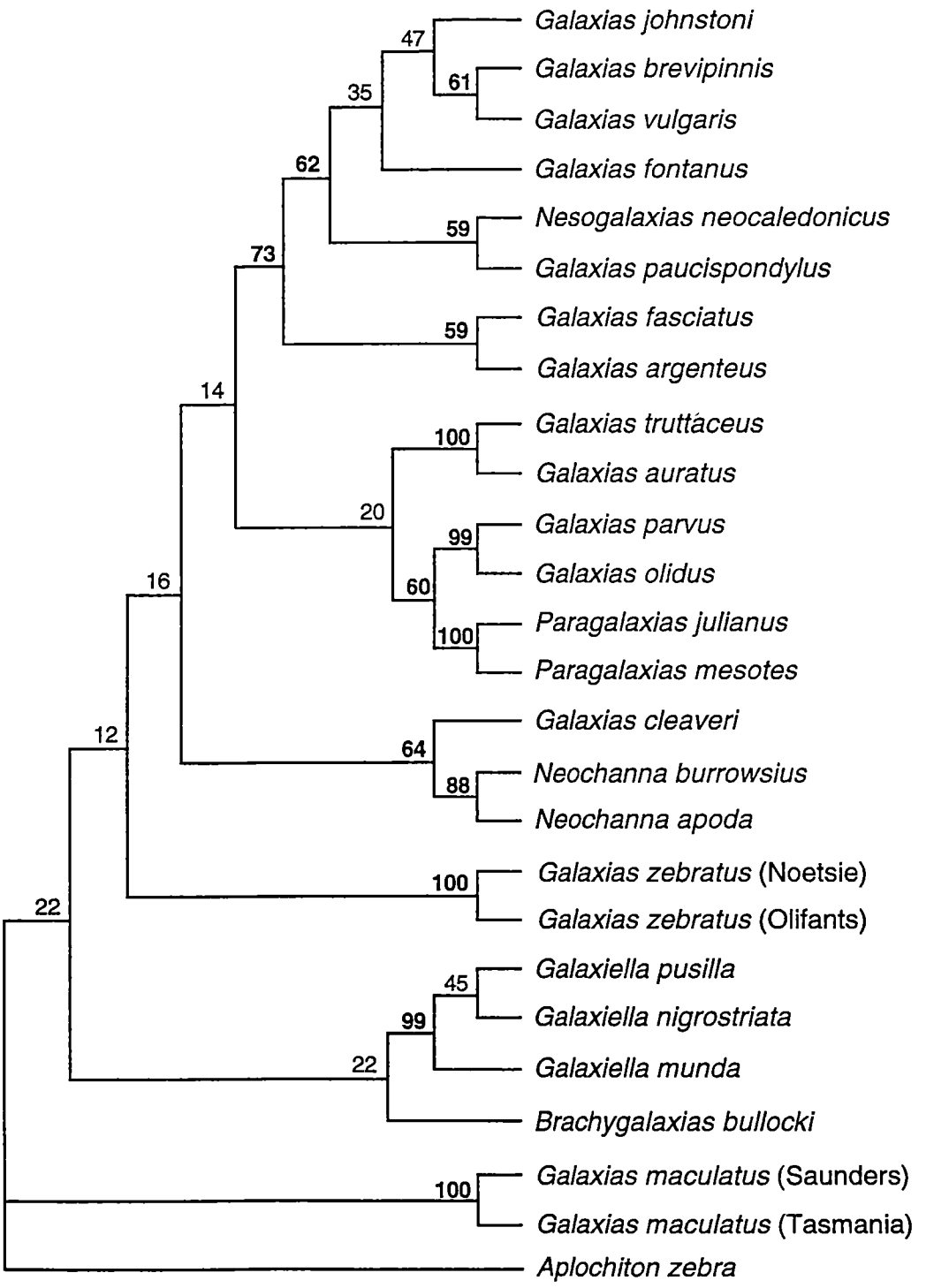

Figure 2.15 Neighbor-Joining bootstrap cladogram based on 16S rRNA sequences. Distances were calculated with the maximum likelihood algorithm of Felsenstein (1993). Values at branch points indicate bootstrap support as based on 500 replicate distance analyses. Values ≥ 50% are shown in bold.

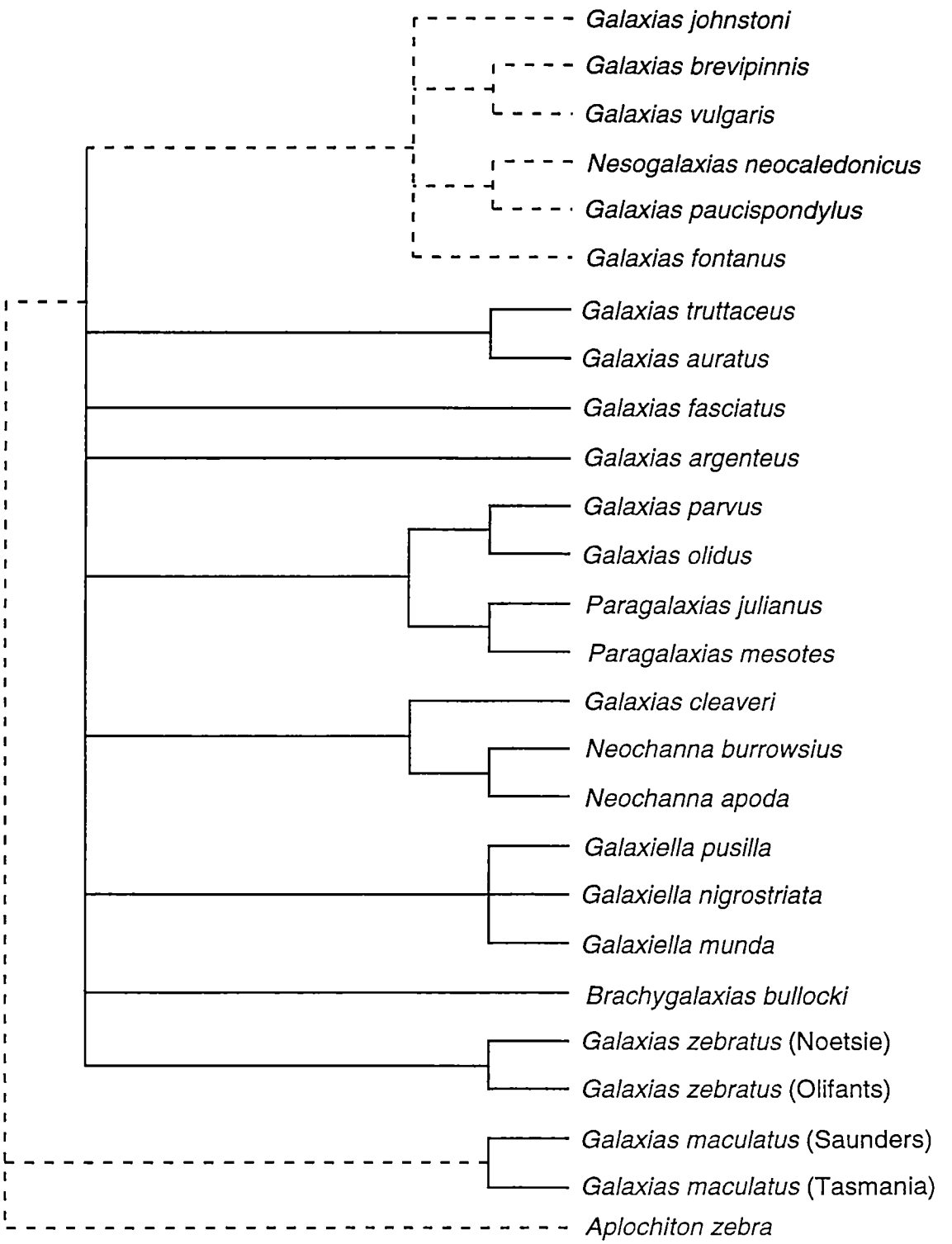

Figure 2.16 A conservative galaxiine 16S rRNA phylogeny, showing only the groupings common to maximum likelihood, maximum parsimony and and neighbor-joining trees. Clades that received low levels of bootstrap support are indicated by dotted lines.

Galaxioid 16S rRNA phylogeny

Four galaxioid 16S rRNA sequences were aligned with CLUSTAL (Table 2.11). Variation was detected at 169 (32%) of the nucleotide positions. Genetic divergences between galaxioid taxa were calculated (Table 2.12) with *G. brevipinnis* as a representative of the Galaxiinae *sensu* Begle (1991). Pairwise divergences ranged from 8.1% (*G. brevipinnis-Aplochiton*) to 28.2% (*G. brevipinnis-Lepidogalaxias*). The *Lepidogalaxias salamandroides* 16S rRNA was highly divergent from other galaxioid sequences, differing by a mean of 27.6%. In contrast, the remaining galaxioid taxa were separated by <20% sequence divergence, with a mean of 13.7%. Of the observed substitutions, 53% were TIs and 47% were TVs, representing a TI bias of 1.1.

For parsimony analysis of the galaxioid sequences, the Galaxiinae were represented by *G. brevipinnis*, *G. argenteus*, and *Nesogalaxias neocaledonicus*. In addition, 16S rRNA sequences for the outgroups *Salmo trutta* (EMBL, X77559) and *Oncorhynchus mykiss* (EMBL, X77564) were aligned with the galaxioid sequences. No osmerid 16S rRNA sequences were included in this analysis because none are presently published in nucleotide databases.

Random treelength distribution was examined to assess the phylogenetic information content of the galaxioid 16S rRNA data. Six sequences, representing salmonid, and five galaxioid lineages were included. The length distribution of 10,000 random trees revealed no significant skew to the left ($g1$ = -0.24, P>0.05), indicating the absence of strong phylogenetic signal in the cytochrome *b* data set (Hillis and Huelsenbeck 1992). However, when only TVs were analysed, a marginally significant phylogenetic information content was revealed ($g1$ = -0.47, 0.01<P<0.05).

Parsimony analysis was performed with a TV/TI weight of 2:1, and also with TIs excluded (Fig 2.17). The mp tree (2:1 weighting) supported the monophyly of the salmonids and Galaxiinae (Fig. 2.17). In addition, *Aplochiton* was grouped with the galaxiines, and *Lovettia* and *Lepidogalaxias* were placed as sister taxa. However, these groupings received low bootstrap support. *Retropinna* was placed as a basal galaxioid with bootstrap values of 52% (2:1) and 38% (TV).

The level support for alternative galaxioid relationships was examined. *Lovettia* was grouped with *Aplochiton* and the galaxiines in 51% (2:1) and 70% (TV) of bootstrap replicates. *Lepidogalaxias* was placed as primitive to other galaxioids with 27% (2:1) and 60% (TV) bootstrap support.

Table 2.11 Partial sequences of the mitochondrial 16S rRNA gene (L-strand) of four galaxioid taxa. Dots indicate matches with the *Aplochiton zebra* sequence. Sequence alignment was performed with CLUSTAL.

Aplochiton zebra	CCTGCCTGCC	CTGTGACTAT	A–GTTTAACG	GCCGCGGTAT	TTTGACCGTG	CAAAGGTAGC
Lovettia sealii	.	.	T C	...	. .	.. .
Retropinna tasmanica	C ...	G . C	GG A .	.	A	G
Lepidogalaxias salamandroides	..	. . ——	TA	. .	. A	. .
Aplochiton zebra	GCAATCACTT	GTCTTTTAAA	TGAAGACCTG	TATGAATGGC	AAGACGAGGG	CTAAGCTGTC
Lovettia sealii	..		AG C	..	.	. A
Retropinna tasmanica	. . .	C.	. .. .	. .	T . .	TGA .
Lepidogalaxias salamandroides	. ... .	... ..	.A. . . C	. .	C A	T A . .
Aplochiton zebra	TCCTCTCCCA	AGTCAGTGAA	ATTGATCTCC	CCGTGCAGAA	GCGGGGATAA	TAACATAAGA
Lovettia sealii	. T.T. C	. . .	.. . .	. . ..	. T	CT ...
Retropinna tasmanica	. .. C	C . ..	.. ..	.. ..	. .. T	CC ....
Lepidogalaxias salamandroides	.T. T	.. . . .	AG .G	... ...	A .AC . C	CCG. .
Aplochiton zebra	CGAGAAGACC	CTATGGAGCT	TTAGACACTA	GACAGCCCAC	GTTAAGAAAG	CTT–––CCCA
Lovettia sealii	.. .	. ... . .	. ..	A. .	. A AC GC	T . TTA
Retropinna tasmanica	..	.	. .	. .	C AC GC	CACC TAG
Lepidogalaxias salamandroides	. .	. . . .. .	A T AG	TTT –A T	AAA ATT C	TCAAATA T
Aplochiton zebra	ACAGTATAAA	CTTGCTGGCA	ACTGATTTAC	TTGTCTTCGG	TTGGGGCGAC	CGCGGGGGAA
Lovettia sealii	G G.A.CT G	.–––––	.T. C .T. C A	A . .	.. .	. . .
Retropinna tasmanica	TAG CCAG	.A.TG. . C	C.. TC CC.	C.	.	. . . A .
Lepidogalaxias salamandroides	.T AG AT .	.ACAG TT	A.. A	.A T .	. .	A .A A
Aplochiton zebra	AAATTAGCCC	CCATGTGGAA	CGGGAGTATA	––CTCCTACA	GCCAAGAACT	ACCGTTCTAA
Lovettia sealii	.G AG .. .	. . C	.. . . T	T––– ...A	TTT ... TA	C .
Retropinna tasmanica	–G AA .. T	... ..	T.A– TAC	CA.CTTC. .	. TC. .G.C	G A C C
Lepidogalaxias salamandroides	. TAA T. T	CA C	T A ––– AC	TC. .T.TA	T A GA . C	..ACA . .
Aplochiton zebra	GCAACAGAAA	TTCTGACCAA	AAATGATCCG	GCA–ACGCCG	ATCAACGGAC	CCAGTTACCC
Lovettia sealii	AA. . ..	.. . ..	.T– ???	?. A –...	.T .A A	. .
Retropinna tasmanica	. G . . .	.. .	... .	.. C . ..	..T ... .A	A ..
Lepidogalaxias salamandroides	AA T.. C C	AG	T.. . .	A.TT AAT.	. .T A.	.A ..
Aplochiton zebra	TAGGGATAAC	AGCGCAATCC	TCTCCCAGAG	TCCCTATCGA	CGAGGGGGTT	TACGACCTCG
Lovettia sealii	. . .	. .. . .	C . .	..A	.. ..	. . ...
Retropinna tasmanica	. . . .	. .	. .	.....	. ..	. . .
Lepidogalaxias salamandroides	....	. . .T	...T	C. T. ...	.A AA .	.. . .
Aplochiton zebra	ATGTTGGATC	AGGACATCCT	AATGGTGCAG	CCGCTATTAA	GGG	
Lovettia sealii	. .			A	.	
Retropinna tasmanica	. .		T	A	.	
Lepidogalaxias salamandroides	... .		. T	T ..	.	

Table 2.12 Percentage sequence divergences (above diagonal) and observed nucleotide substitutions (below diagonal) between galaxioid16S rRNA sequences. Numbers in brackets are observed transversion substitutions. Divergence values were calculated with the maximum likelihood algorithm of Felsenstein (1993).

Taxon	*Galaxias*	*Aplochiton*	*Lovettia*	*Lepidogalaxias*	*Retropinna*
Galaxias brevipinnis	–	8.1	10.1	28.2	17.6
Aplochiton zebra	39 (16)	–	11.8	25.7	15.7
Lovettia sealii	47 (20)	54 (28)	–	25.3	18.7
Lepidogalaxias salamandroides	116 (54)	107 (51)	116 (46)	–	31.1
Retropinna tasmanica	79 (39)	71 (36)	81 (41)	126 (60)	–

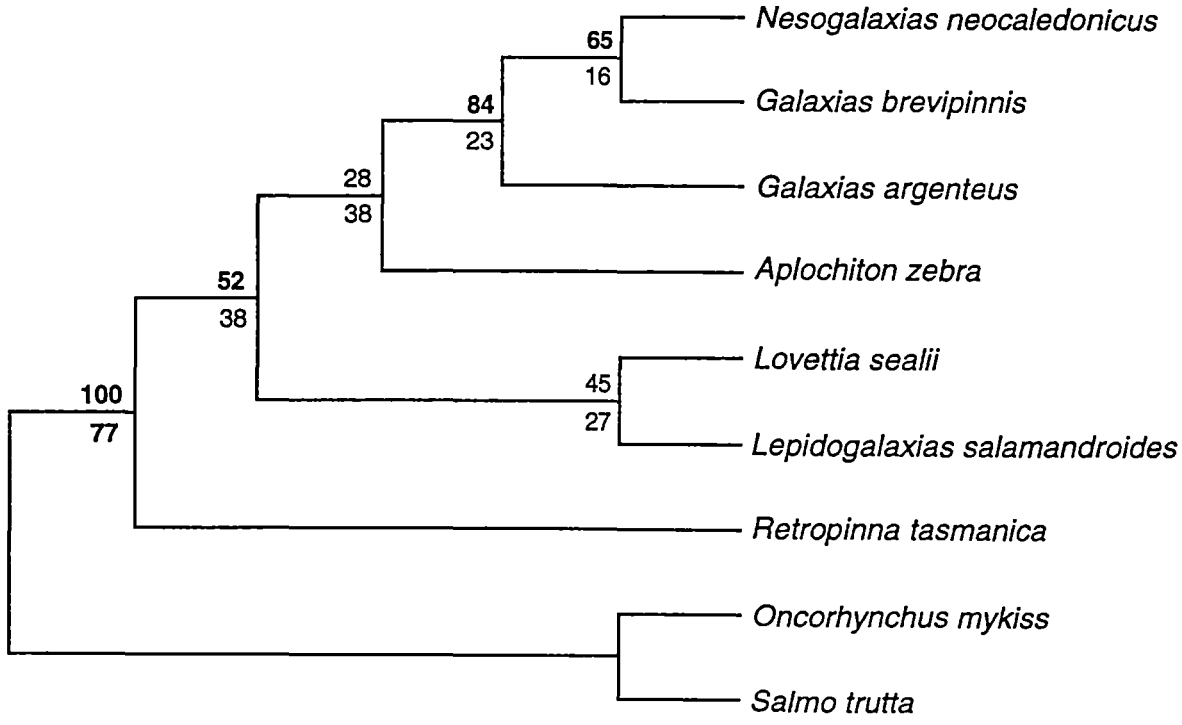

Figure 2.17 Single mp tree (369 steps) for galaxioid 16S rRNA sequences. Two salmonid taxa were included as outgroups. Bootstrap values above nodes are for analysis with TV:TI weight of 2:1. Values below nodes are for analysis of TVs only. Estimates ≥ 50% are shown in bold.

2.3.3 Combined mtDNA sequence phylogeny of the galaxiines

Character congruence between the cytochrome *b* and 16S rRNA data sets was assayed by the Mickevich-Farris index (Table 2.13). The lowest Mickevich-Farris index (1.29 $x10^{-2)}$ and hence the maximum level of congruence was attained with a TV:TI cost ratio of 2:1. Similar levels of congruence were attained with unweighted data, a TV:TI cost ratio of 4:1, and EOR weighting. However, exclusion of the transition data resulted in a minimum of character congruence between the data sets ($2.08\ x10^{-2}$).

Table 2.13 Character congruence as measured by the Mickevich-Farris extra-steps index. The mp tree-lengths for the cytochrome *b*, 16S rRNA and combined sequence data sets are shown. These are used to determine the number of extra steps forced on the combined data, and the M-F index ($x10^{-2}$), for different weighting strategies.

Weighting	Cytochrome b (a)	16S rRNA (b)	Combined data (ab)	(ab - (a + b))	M-F index (x 10-2)
Unweighted	450	737	1204	17	1.41
TV:TI = 2	605	924	1549	20	1.29
TV:TI = 4	910	1288	2227	28	1.30
TV only	152	177	336	7	2.08
EOR	591	786	1396	19	1.36

The combined molecular analysis of the Galaxiinae included 27 sequences, each 825 base pairs in length, representing over 22 kilobases of data. Maximum likelihood analysis of the combined 16S rRNA and cytochrome *b* data sets recovered a ML tree with a log likelihood of -7182.6 (Fig. 2.18). All of the ML tree branch lengths were significantly positive. The ML tree supported a number of accepted clades including *Galaxiella*, *Paragalaxias*, *Neochanna*, [*G. fasciatus*, *G. argenteus*] and [*G. truttaceus*, *G. auratus*]. As in the separate gene analyses, *G. olidus* and *G. parvus* formed a clade which was in turn united with *Paragalaxias*, and the clade [*Nesogalaxias*, *G. paucispondylus*, *G. brevipinnis*, *G. vulgaris*, *G. fontanus*, *G. johnstoni*] was supported. In addition, *G. cleaveri* was grouped with *Neochanna*, *Galaxiella* was grouped with *Brachygalaxias*, and *G. maculatus* was placed as a basal galaxiine.

The topology of the NJ bootstrap tree (mp bootstrap values are also shown, Fig. 2.19) was similar to the ML tree topology. Areas of incongruence were restricted to clades that received low or inconsistent bootstrap values. For example, unlike the ML tree, the mp and NJ trees placed *G. zebratus* as the sister of *Galaxiella* and *Brachygalaxias*, albeit at low levels of support. Furthermore, the arrangement of taxa within [*Nesogalaxias*, *G. paucispondylus*, *G. brevipinnis*, *G. vulgaris*, *G. fontanus*, *G. johnstoni*] was poorly supported.

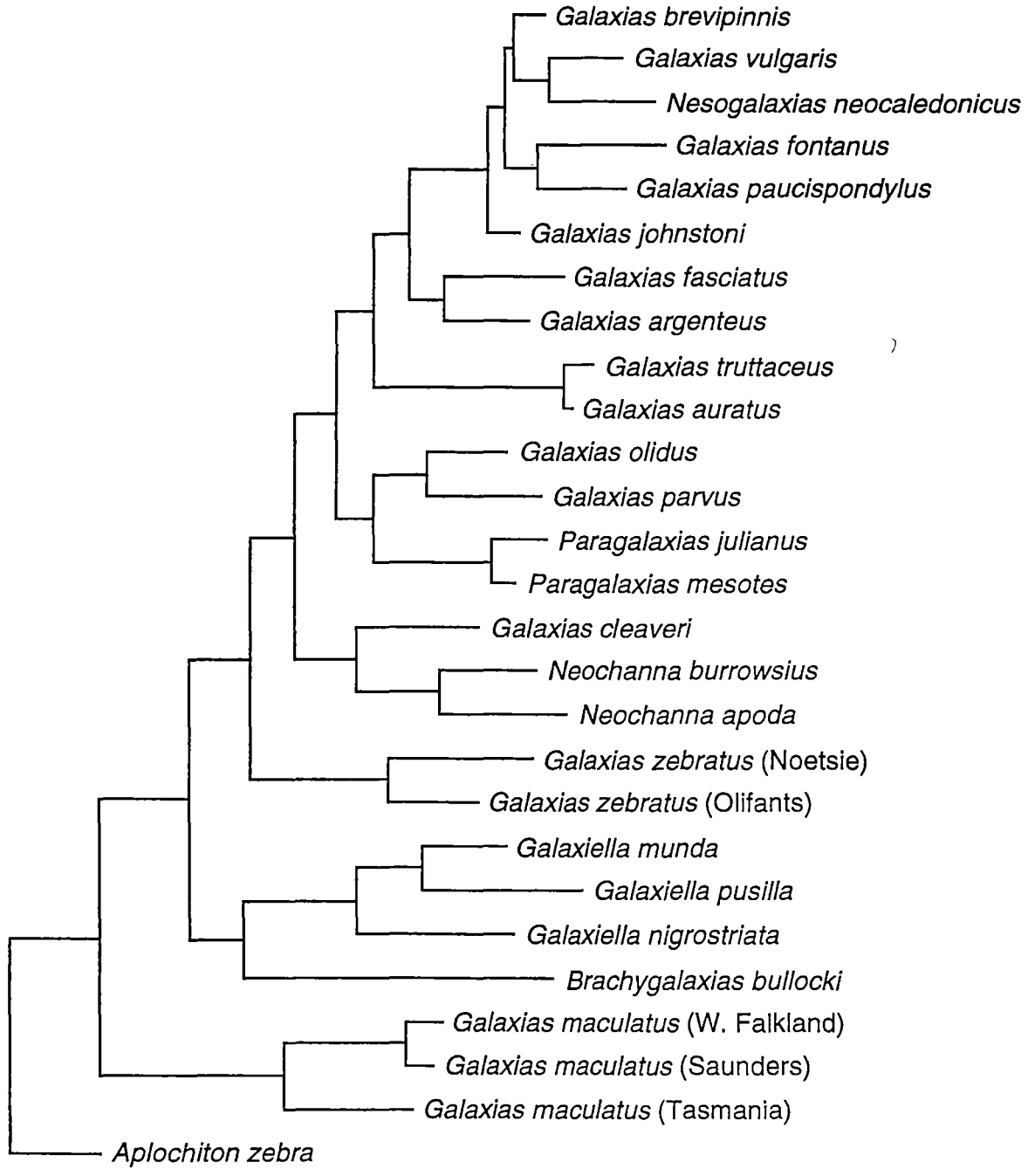

Figure 2.18 Maximum likelihood tree based on combined cytochrome *b* and 16S rRNA sequences. Branch lengths are proportional to the expected number of nucleotide substitutions. The analysis was performed with an expected TV/TI ratio of 2:1. The log likelihood of the tree is -7182.6.

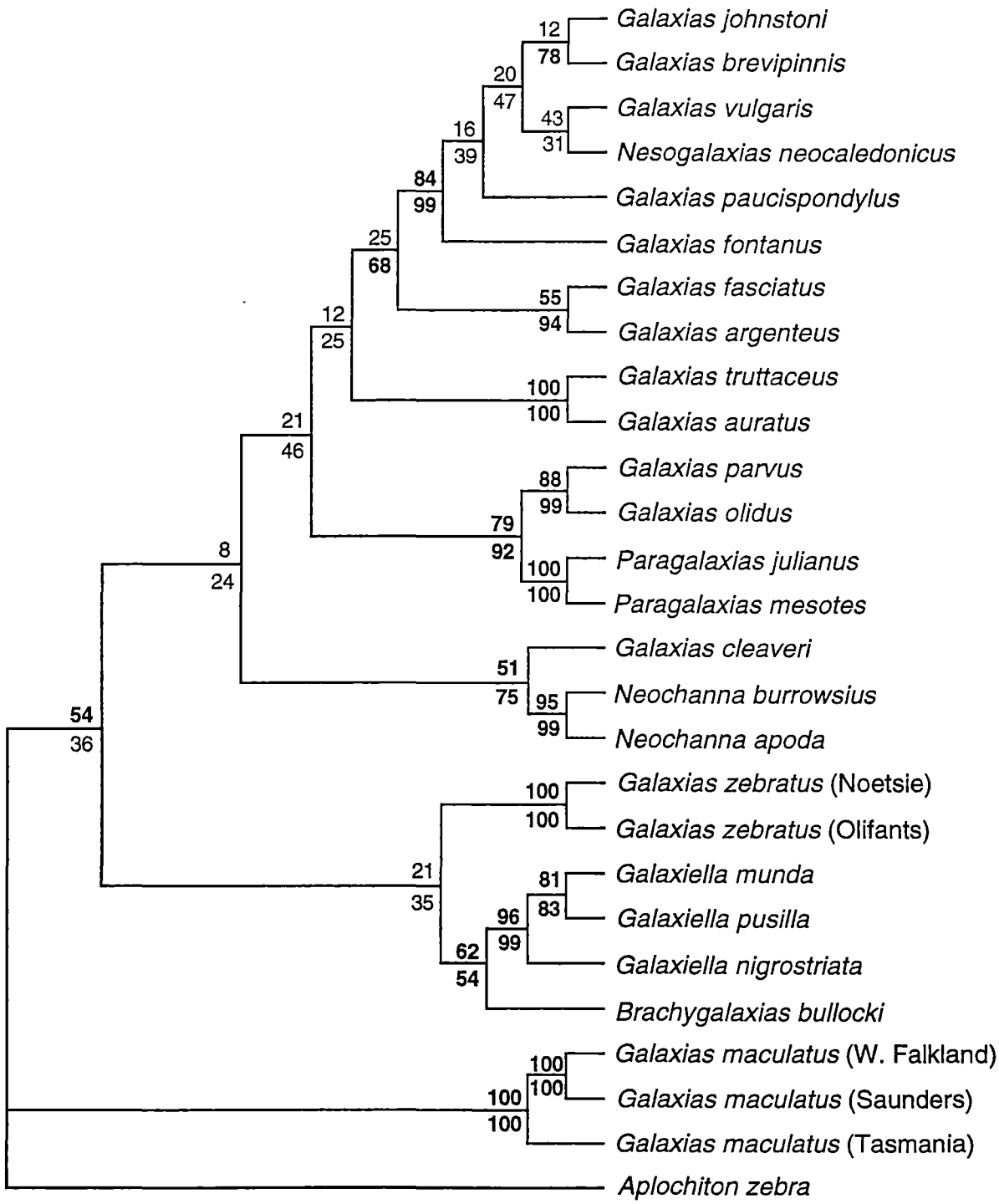

Figure 2.19 Galaxiine phylogeny from analysis of combined cytochrome *b* and 16S rRNA sequences. Values at branch points indicate bootstrap support as based on 500 resamplings of the data (TV/TI = 2:1). Values above nodes are from parsimony analysis while those below nodes are for distance analysis. Bootstrap estimates ≥ 50% are shown in bold.

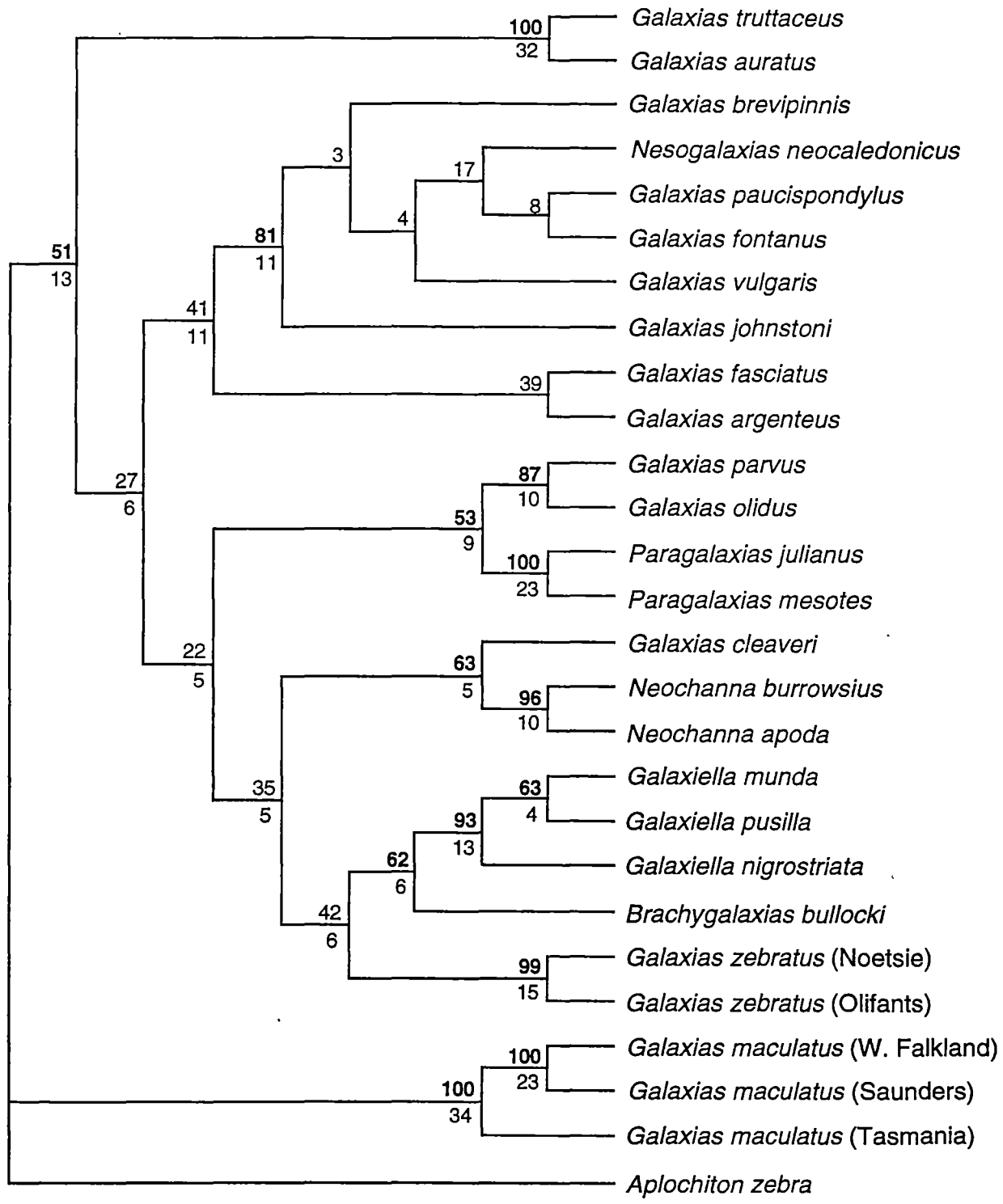

Figure 2.20 Galaxiine phylogeny from analysis of combined cytochrome *b* and 16S rRNA sequences with EOR weighting. Values above branch points are bootstrap percentages (500 replicates); estimates ≥ 50% are shown in bold. Numbers below nodes are Bremer support values >3 steps.

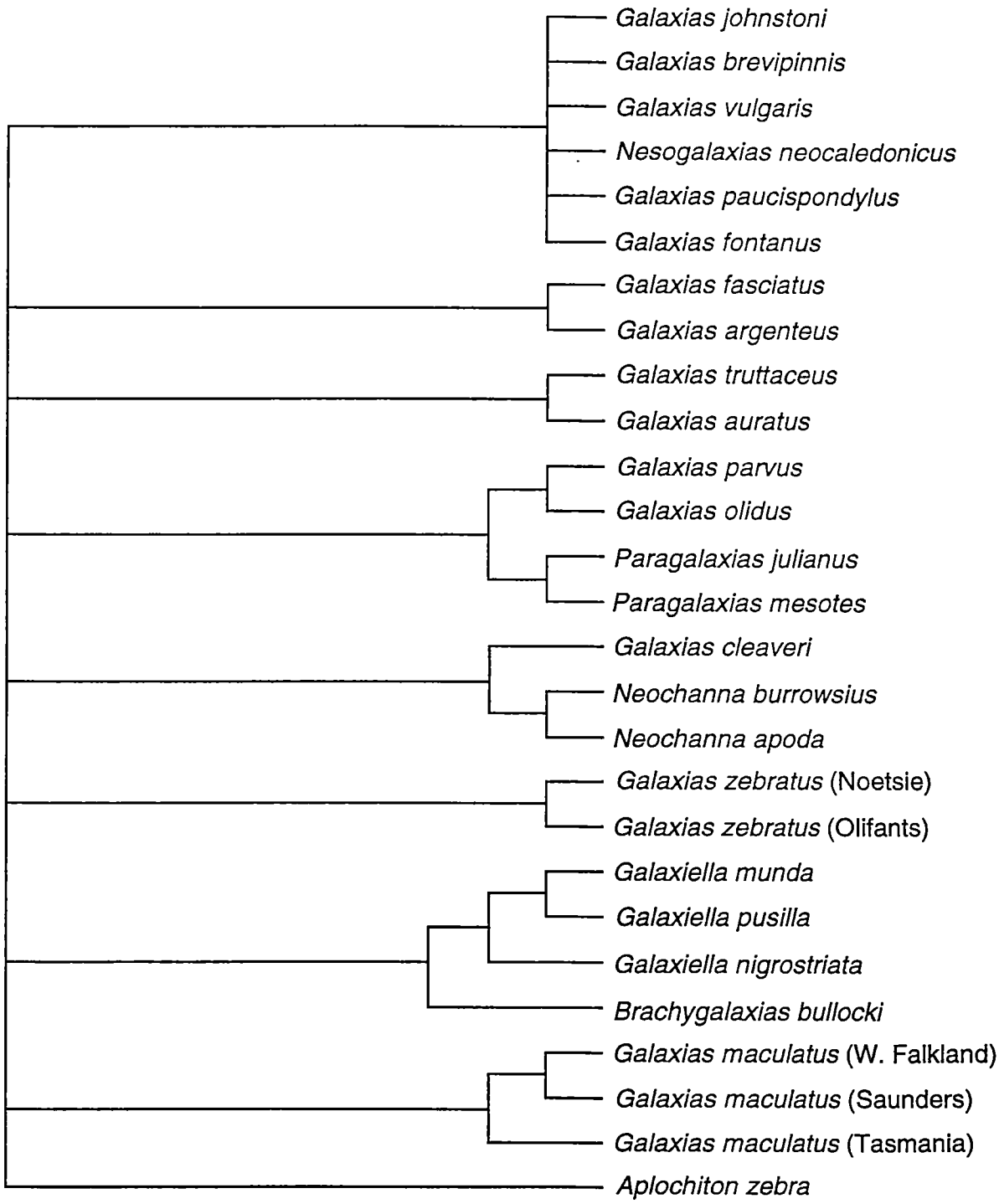

Figure 2.21 A conservative galaxiine phylogeny based on the combined cytochrome *b* and 16S rRNA data sets. Clades that did not receive a substantial level of bootstrap support from both maximum parsimony (PAUP) and distance (neighbor-joining) analysis of the combined data were collapsed to yield polytomies.

The mp and NJ analyses produced almost identical levels of bootstrap support for several clades (Fig. 2.19). However, the NJ method afforded substantially more support for [*G. cleaveri*, *Neochanna*] (75% as against 51%), [*G. fasciatus*, *G. argenteus*] (94% versus 55%), and [*G. brevipinnis*, *G. johnstoni*] (78% versus 12%). Conversely, parsimony analysis supported *G. maculatus* as a basal galaxiine with a bootstrap of 54%, as opposed to 36% with the NJ method.

The EOR weighting strategy, implemented in PAUP with two stepmatrices, produced a single mp tree 1396 steps long (Fig 2.20). This topology was similar to the 2:1 bootstrap tree, the only differences involving the configuration of weakly supported basal nodes, and the arrangement of species within the clade [*Nesogalaxias*, *G. paucispondylus*, *G. brevipinnis*, *G. vulgaris*, *G. fontanus*, *G. johnstoni*]. Bootstrap analysis with EOR weights produced similar levels of support to the 2:1 weighting method. However, there was diminished support for the [*G. fasciatus*, *G. argenteus*] clade (39%) and for the [*Paragalaxias*, *G. parvus*, *G. olidus*] clade (53%). The placement of *Galaxias zebratus* as sister to [*Brachygalaxias*, *Galaxiella*] received higher support (42%), but this value is still low. Clades with high bootstrap values typically received high levels of Bremer support. Specifically, groups that received bootstrap proportions over 90% had Bremer support of at least 10 steps. Similarly, the weakly supported groupings within [*Nesogalaxias*, *G. paucispondylus*, *G. brevipinnis*, *G. vulgaris*, *G. fontanus*, *G. johnstoni*] received Bremer values <4.

A conservative mtDNA sequence phylogeny of the Galaxiinae is shown in Fig. 2.21. In this tree, only clades that received at least 50% bootstrap support from both mp and NJ analysis (Fig. 2.19) are resolved.

2.3.4 Combined mtDNA sequence phylogeny of the galaxioids

The combined galaxioid cytochrome *b* and 16S rRNA sequences were analysed with maximum parsimony methods with two weighting strategies. Firstly, a TV:TI cost ratio of 2:1 was used. Alternatively, TIs were excluded, with first and second codon positions given double the weighting of third positions. The TV parsimony tree (Fig 2.22) supported [*Lovettia*, *Aplochiton*, galaxiines], united *Retropinna* with the osmerids, and placed *Lepidogalaxias* as basal to other osmeroids.

The monophyly of the galaxiines, osmerids and salmonids was supported by high bootstrap values with both weighting strategies (Fig. 2.22). In addition, *Aplochiton zebra* was placed in a galaxiid clade with bootstraps >50% for both methods. *Lepidogalaxias* was placed as basal to the Osmeroidei in 53% of TV parsimony bootstrap replicates. The phylogenetic positions of other galaxioids were supported by

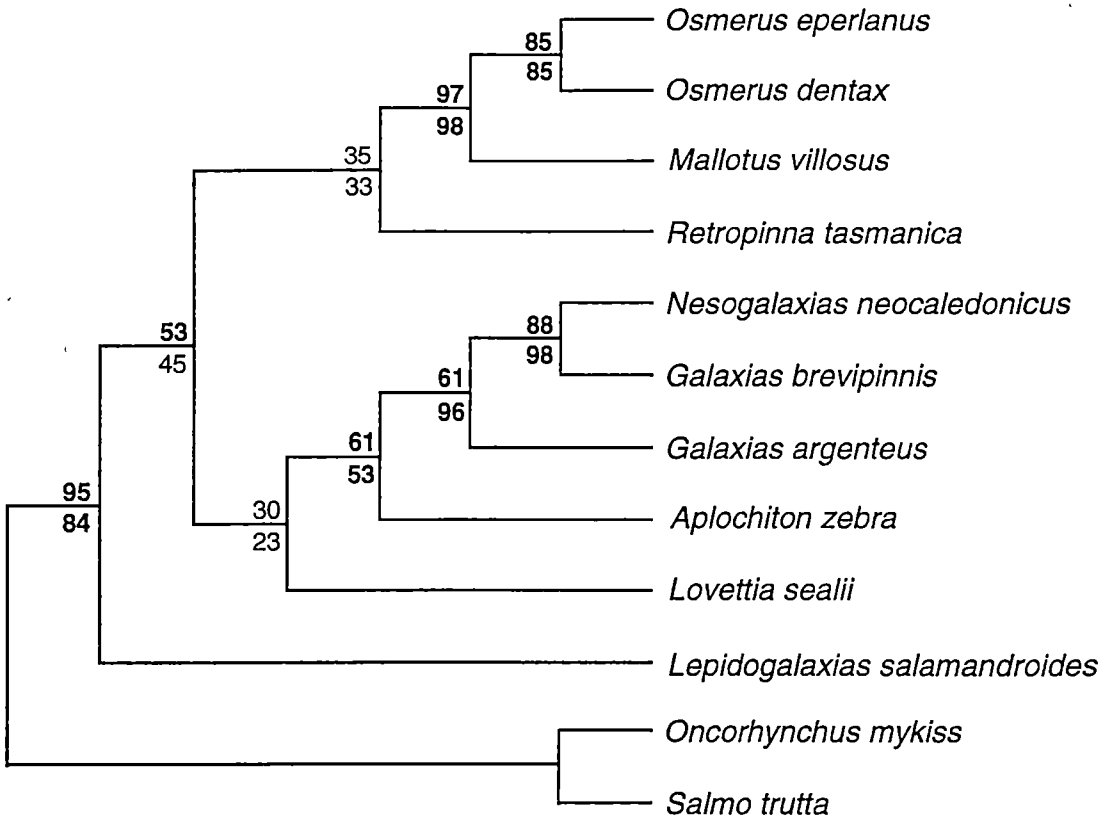

Figure 2.22 The mp tree (307) steps from TV analysis of combined galaxioid cytochrome *b* and 16S rRNA sequences. Two salmonids and three osmerids were also included. Values at branch points indicate bootstrap support (500 resamplings), values above nodes are for TV parsimony, with first and second codon positions given a weighting of 2. Values below nodes are for TV:TI weighting of 2:1; estimates ≥ 50% are shown in bold.

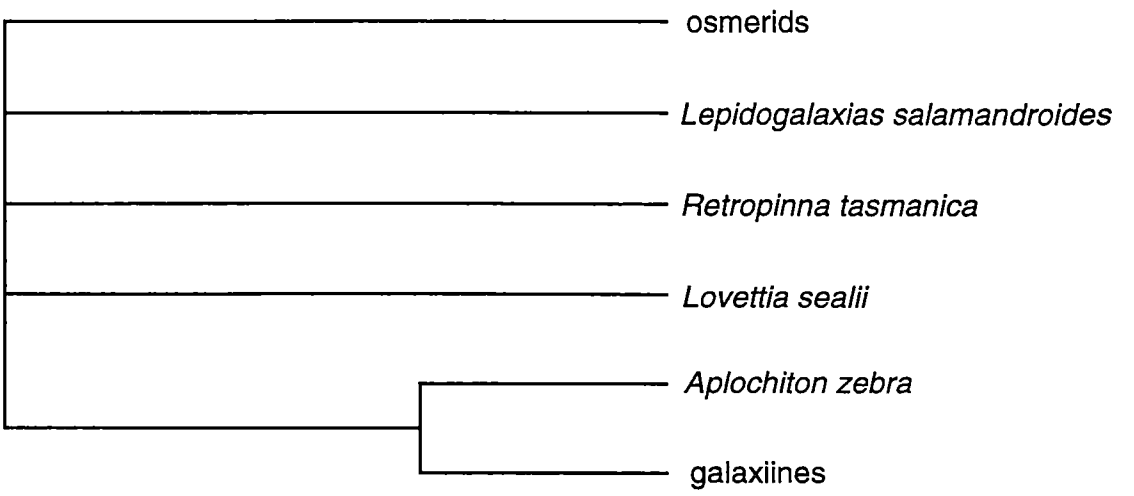

Figure 2.23 A conservative phylogeny of the osmeroids based on the combined molecular data sets. Clades that did not receive a substantial level of bootstrap support from both weighting stategies were collapsed to yield polytomies.

very low bootstrap estimates. *Lovettia* was placed as a sister of *Aplochiton* and the Galaxiinae (30% in TV analysis). Alternatively, *Lovettia* was placed as the sister of *Lepidogalaxias* with 7% (TV) and 13% (2:1) support. The clade [*Lepidogalaxias, Lovettia, Aplochiton*, galaxiines] received 12% support with 2:1 weighting. The monophyly of the Aplochitonidae *sensu* McDowall (1969; [*Lovettia, Aplochiton*]) received bootstraps of only 1% (TV) and 2% (2:1). Similarly, the monophyly of the Galaxioidea was weakly supported (<2%).

Apart from the monophyly of the galaxiines and the osmerids, the only well supported clade was the grouping of *Aplochiton* with the galaxiines (Fig. 2.23).

2.4 Discussion

Intraspecific versus interspecific divergence

Intraspecific cytochrome *b* sequence divergences are typically about 1% in fishes (e.g. Taylor and Dodson 1994; Patarnello *et al.* 1994; Finnerty and Block 1992; McVeigh *et al.* 1991; Meyer *et al.* 1990). Until the current study, the 3% divergence reported in *Gasterosteus aculeatus* (Orti *et al.* 1994) was the highest intraspecific value for fish cytochrome *b*. However, high sequence divergence estimates based on restriction enzyme analysis of mtDNA have been reported for isolated populations of non-diadromous fishes. For example, Bermingham and Avise (1986) reported up to 8.7% divergence between geographic isolates of *Lepomis*. Similarly, estimates of 7.4% and 8.8% divergence have been reported for isolated *Gadopsis bispinosus* (Ovenden *et al.* 1988; Waters *et al.* 1994) and 9.8% between populations of *Gadopsis marmoratus* (Ovenden *et al.* 1988). It should be noted that these RFLP analyses sample the whole mitochondrial genome, including some highly variable regions. It is therefore expected that mtDNA RFLP studies will reveal more variation than is detected by the direct sequencing of conserved parts of the genome.

Estimates of sequence divergence between the Tasmanian and Falklands *G. maculatus* cytochrome *b* sequences averaged 18.8%, with a maximum of 19.2%. Moreover, estimates of sequence divergence between the Olifants and other *G. zebratus* populations averaged 13.2%, with a maximum of 13.8%. These levels are considerably larger than other intraspecific values reported for both cytochrome *b* and the whole mitochondrial genome (see above). The intraspecific divergence estimates for *G. zebratus* and *G. maculatus* are comparable with interspecific and intergeneric comparisons within the osmeroids, the sister group to the galaxioids. Specifically, they exceed reported interspecific divergences for *Osmerus* (5-9%) and are more comparable with the intergeneric divergence between *Mallotus* and *Osmerus* (about 15%; Taylor and Dodson 1994).

These intraspecific divergences are high in comparison with those detected between morphologically distinct galaxiine species. For example, *Paragalaxias* species are separated by 7.6% divergence, while *G. truttaceus* and *G. auratus* differ by only 2.1%. In addition, the taxa *G. brevipinnis*, *G. johnstoni*, *G. vulgaris* and *Nesogalaxias neocaledonicus* are separated from each other by a mean of 9.0% cytochrome *b* sequence divergence, with a maximum of 10.6%. In fact, the intraspecific values are more comparable with divergences such as 13.7% between *G. fasciatus* and *G. argenteus*, 14.6% between *G. olidus* and *G. parvus*, and 13.6% between *Neochanna apoda* and *N. burrowsius*.

Similarly, a considerable amount of 16S rRNA sequence divergence was detected within both *G. maculatus* (maximum 5.6%) and *G. zebratus* (5.3%). While these intraspecific divergences are lower than those for the corresponding cytochrome *b* sequences, this is consistent with the relatively low number of variable sites detected in the 16S rRNA gene. These levels of intraspecific divergence exceed interspecific 16S rRNA divergences within the Galaxiinae. For instance, 1.0% was detected between *G. truttaceus* and *G. auratus*, 2.8% between *G. olidus* and *G. parvus*, and 3.5% between *G. fasciatus* and *G. argenteus*. They are also greater than interspecific divergences (mean 3.0%, maximum 4.2%) within the clade [*Nesogalaxias*, *G. paucispondylus*, *G. brevipinnis*, *G. vulgaris*, *G. fontanus*, *G. johnstoni*]. In fact, the divergences within *G. zebratus* and *G. maculatus* are similar to those between *N. apoda* and *N. burrowsius* (5.0%), and *Galaxiella pusilla* and *G. munda* (5.5%).

These high levels of intraspecific cytochrome *b* and 16S rRNA divergence may be interpreted in a number of ways. Firstly, there may be as yet unrecognised morphological species within the *G. zebratus* and *G. maculatus* "complexes". Secondly, it may be that the ratio of morphological evolution to mtDNA evolution varies markedly between different galaxiine taxa. Such decoupling of molecular and morphological evolution has been suggested for other taxonomic groups. For example, Avise *et al.* (1994) noted that despite the rapid loss of shell living habit in king crabs (Cunningham *et al.* 1992), 16S rRNA sequence evolution was probably slower in this group than in the morphologically conserved horseshoe crabs. Thirdly, the high levels of intraspecific diversity in *G. zebratus* and *G. maculatus* could be explained by an unusually rapid rate of molecular evolution in these taxa. However, the low divergence (0.3%) between Krom and Kouga River *G. zebratus* cytochrome *b* sequences suggests that no such rapid molecular evolution has occurred, as these genetically similar populations have probably remained isolated since a period of low sea level during the Pleistocene. The first two explanations (cryptic species and/or slow morphological

evolution) appear to be more plausible explanations of the high intraspecific divergences.

Galaxiine phylogeny

The observed TI:TV bias for pairwise comparisons of galaxiine sequences (between 2:1 and 3:1 for both genes) is lower than those reported in other taxonomic groups. For example, reported biases for fish cytochrome *b* include 11:1 in *Osmerus* (Taylor and Dodson 1994) and 7:1 in *Salmo* (Patarnello *et al.* 1994) and *Gasterosteus* (Orti *et al.* 1994). The linear relationships detected between sequence divergence and both TIs and TVs for both genes suggest that the sequences are not saturated with TIs at divergences of less than 20% for cytochrome *b*, and less than 15% for 16S rRNA sequences.

The cytochrome *b* and 16S rRNA data sets provided similar levels of phylogenetic resolution within the Galaxiinae. Specifically, the separate cytochrome *b* and 16S rRNA analyses both provided substantial support for nine clades. Seven of the nine were common to both analyses, while the additional groupings were not conflicting. Thus the conservative findings from the separate analyses are completely congruent. In addition, clades that received strong support from only one of the separate molecular data sets were generally supported (albeit weakly) by the other data analysis. Such clades include [*G. cleaveri*, *Neochanna*], [*G. parvus*, *G. olidus*] and [*Nesogalaxias*, *G. paucispondylus*, *G. brevipinnis*, *G. vulgaris*, *G. fontanus*, *G. johnstoni*]. These findings suggests that, despite their different evolutionary properties, mitochondrial cytochrome *b* and 16S rRNA genes are concordant and represent a single phylogeny.

The greatest phylogenetic resolution within the Galaxiinae was obtained with the combined molecular analysis, which provided substantial support for 13 clades (not including the Saunders and West Falkland *G. maculatus* clade). Generally, the combined data produced higher levels of bootstrap and Bremer support than were obtained from the separate analyses. Notably, the combined data provided substantial support for [*Galaxiella*, *Brachygalaxias*] and [*G. fasciatus*, *G. argenteus*], clades which received only weak support in the separate gene phylogenies. Levels of bootstrap support for most of the well supported clades were not unduly affected by most weighting strategies. However, tests of character congruence indicated that total exclusion of TI information was a poor option to take with the data.

Phylogenetic resolution was limited to relatively closely related galaxiine taxa with basal groupings always receiving low bootstrap support. Taxa within strongly supported cytochrome *b* clades were separated by a maximum of 21.7% sequence divergence (between *Galaxiella pusilla* and *G. nigrostriata*) and a mean of 13.2%. For the 16S

rRNA sequence analysis, the highest pairwise divergence between taxa within well supported clades was 7.2%, again between *G. pusilla* and *G. nigrostriata* (mean 4.6%). However, when sequence data were combined, the deeper [*Galaxiella*, *Brachygalaxias*] clade was resolved. The divergences between these genera averaged 26.0% for cytochrome b and 14.4% for 16S rRNA sequences.

The lack of phylogenetic resolution at various levels within the Galaxiinae may be due to a number of factors. These include saturation effects, rate heterogeneity, rapid cladogenesis and lineage sorting. Saturation may have occurred between distantly related galaxiine sequences, preventing the resolution of deep phylogenetic splits. The fact that some sequences (e.g. *G. zebratus*, *G. maculatus*) differ from those of other species by over 30% corrected divergence suggests that they may have reached saturation. Graybeal (1993) noted that because amino acid replacements are rare, 25% (about 30% corrected) divergence represents the approximate saturation level for cytochrome *b*.

Variation in the rate of sequence evolution may also be a factor hindering phylogenetic resolution. Rate heterogeneity can confound parsimony methods and lead to the artificial clustering of rapidly evolving lineages (Larson 1991). Within the Galaxiidae, there is some evidence for unequal rates of molecular evolution between taxa. Assuming that *Aplochiton* is the nearest common ancestor of the galaxiines, this species would be expected to have the most divergent galaxiid sequence. However, for both 16S rRNA and cytochrome *b* sequences, the highest divergences were obtained for pairwise comparisons involving species such as *Brachygalaxias bullocki* and *G. maculatus*. This may reflect rate heterogeneity, or possibly saturation of divergent sequences. In addition, there is possible evidence of rate heterogeneity within the clade [*Nesogalaxias*, *G. paucispondylus*, *G. brevipinnis*, *G. vulgaris*, *G. fontanus*, *G. johnstoni*]. The most divergent 16S rRNA sequences within this group are *G. johnstoni* and *N. neocaledonicus* (4.2%). In contrast, for cytochrome *b*, the maximum divergence is between *G. fontanus* and *N. neocaledonicus* (15.3%) while *G. johnstoni* and *N. neocaledonicus* are much more similar, differing by only 9.1%. The 0.0% divergence between *Paragalaxias* 16S rRNA sequences (compared with 1.0% between *G. truttaceus* and *G. auratus*) is surprisingly low given that the *Paragalaxias* cytochrome *b* sequences differ by 7.6% (versus 2.1% for *G. truttaceus* and *G. auratus*). This lack of 16S rRNA divergence may reflect rate heterogeneity for these taxa. Alternatively, it may represent contamination of a *Paragalaxias* 16S rRNA PCR reaction with the DNA of the other paragalaxiid species. However, such contamination is unlikely given the strict adherence to PCR hygiene in this study.

Rapid cladogenesis may have inhibited phylogenetic resolution within the Galaxiinae. For example, within the morphologically diverse clade [*Nesogalaxias*, *G. paucispondylus*, *G. brevipinnis*, *G. vulgaris*, *G. fontanus*, *G. johnstoni*], genetic divergences are relatively low. It may be that this group represents a rapid succession of phylogenetic splits. The resultant short internodes provide relatively few character changes from which to map a phylogeny. A similar explanation has been proposed to account for the lack of resolution in avian molecular phylogenetics. Specifically, Avise *et al.* (1994) noted considerable difficulties in using cytochrome *b* sequences to resolve ancient but closely spaced nodes.

Alternatively, lineage sorting may have resulted in incongruent 16S rRNA and cytochrome *b* gene trees for the *G. brevipinnis* clade. Most taxa are characterised by an amount of intraspecific genetic diversity. Stochastic lineage sorting and extinction can confound phylogenetic analysis (Doyle 1992), particularly when intraspecific diversity is high and bifurcations occur in rapid succession (Wu 1991). Even though mtDNA is a better phylogenetic tool than nuclear DNA under these circumstances (Moore 1995), lineage sorting may still confound mitochondrial sequence phylogenies.

Galaxioidea

Graybeal (1993) noted that, irrespective of mutation rate, pairwise sequence divergences are unlikely to exceed about 25% (30% corrected) for cytochrome *b* because of evolutionary constraints on amino acid sequence. It is therefore likely that the mean divergence of 31.7% detected between galaxioid cytochrome *b* sequences represents saturation. This suggestion is reinforced by the finding that these sequences contain no significant phylogenetic signal at the interfamilial level. Essentially, they are randomised with respect to phylogenetic history (Hillis and Huelsenbeck 1992). Similarly, given the weak phylogenetic signal detected in galaxioid 16S rRNA, the mean divergence between galaxioid 16S rRNA sequences (19.2%) may also represent saturation.

For both cytochrome *b* and 16S rRNA data sets, *Lepidogalaxias salamandroides* is easily the most divergent member of the Galaxioidea. This may reflect rate heterogeneity, or alternatively it may indicate that this species does not belong with the galaxioids. The placement of *Lepidogalaxias* as basal to the Osmeroidei in the combined analysis provided some evidence for the latter hypothesis. However, the bootstrap values for this placement (53% and 45%) are not high.

Unfortunately, the lack of phylogenetic resolution makes it impossible to choose between alternative hypotheses of galaxioid phylogeny. There is support for the close

relationship of *Aplochiton* and the galaxiines as hypothesised by Begle (1991) and Johnson and Patterson (1995). However, the various placements of *Lovettia* suggested by McDowall (1969), Begle (1991) and Johnson and Patterson (1995) all received low levels of bootstrap support. Rosen (1974) suggested that the galaxioids are paraphyletic, with retropinnids and prototroctids derived from osmerids. The combined analysis suggests that this hypothesis may be correct, but bootstrap support for this clade is inconclusive.

Ideally, an increased number of galaxioid and other euteleost taxa are required in a more comprehensive phylogenetic analysis of the Galaxioidea. It is recommended that a gene well suited for the examination of ancient divergences be selected for such a study. Graybeal (1994) found that mitochondrial genes provide relatively little resolution for divergences more than 80 million years old. She suggested nine nuclear protein coding genes that have the potential to resolve phylogenetic splits that occurred up to 200 mya.

CHAPTER 3

Morphological Systematics of the Galaxiinae

3.1 Introduction

There have been several morphological studies of the Galaxiinae. Frankenberg (1969) conducted a morphometric, meristic and osteological study of this group. Similarly, McDowall (1969, 1970, 1971b, 1973b, c, 1978a), Andrews (1976), McDowall and Fulton (1978) and McDowall and Frankenberg (1981) published a considerable number of morphological and osteological observations on the galaxiines. As yet, however, there has been no parsimony analysis of these data.

In what McDowall (1984) described as the only "strictly phylogenetic" interpretation of galaxiine relationships, Rosen (1978) suggested that the slightly forward dorsal fin position of *Galaxias zebratus* is a primitive condition. Furthermore, he suggested that this feature indicates that *G. zebratus* is ancestral to other galaxiines. This was not a rigorous phylogenetic study but rather a hypothesis based on a single character of which the states were misinterpreted. He was apparently unaware of the forward position of the dorsal fin in members of *Paragalaxias*. McDowall (1980, 1984) dismissed Rosen's hypothesis as an attempt that "achieved nothing".

Rosen (1978) and Craw (1979) criticised McDowall for attempting no phylogenetic analysis of the Galaxiinae. In response, McDowall (1980) argued that "until satisfactory revisionary studies of the family have been carried out" any such attempts would be fruitless. Subsequently, McDowall is apparently satisfied with his (and others') revision of the galaxiines, but there has been no attempt on his part to establish a phylogeny for the group. He stated that there are "few readily evident characters that are indicative of major phyletic lineages" (McDowall 1984) and called for new lines of phylogenetic evidence such as DNA hybridisation data.

McDowall (1984) noted that galaxiine features are conserved, species generally lacking specialisation. Elsewhere, McDowall and Frankenberg (1981) described the galaxiines as morphologically plastic, with some characters such as the ethmoid region "prone to rapid modification". Variable characters such as laterosensory pores and pelvic fin ray counts were dismissed as uninformative because derived states "have occurred in widely divergent phylogenetic stocks". Furthermore, McDowall (1984) rejected the inclusion of *Galaxiella* in the genus *Brachygalaxias* as confusing the "understanding of relationships". Such statements seem surprising given that he admitted to having no hypothesis of galaxiine phylogeny. It seems that McDowall assumed *a priori* that all distinct groups of non-diadromous galaxiines represent independent landlocking

events. Thus any character states shared by these groups are thought to be independently derived and thus phylogenetically uninformative.

The osmeroid fishes are characterised by reductive evolution (Fink and Weitzman 1982; Fink 1984; Begle 1991). That is, many differences between species are due to the loss of primitive features rather than the acquisition of new unique character states. Begle (1991) included a considerable number of reductive characters in his phylogenetic analysis of the osmeroids. He emphasised the importance of including all available evidence, including reductive and non-reductive characters. In addition, both homoplastic and uniquely derived synapomorphies can provide support for real clades. As an example, Begle (1991) noted that "limbs have been lost more than once in tetrapods, yet their absence is diagnostic of the monophyletic subgroups snakes and gymnophonians".

McDowall (1970, 1973b, c, 1978a) and McDowall and Frankenberg (1981) were apparently unsuccessful in their search for non-reductive and non-homoplastic characters from which to infer a galaxiine phylogeny. Perhaps a more open-minded approach will prove more fruitful. Through consideration of a wide range of evidence, some phylogenetic signal may be detected from morphological characters.

3.2 Materials and Methods

The collection details of specimens cleared and stained for osteological analysis are shown in Table 3.1. Osteological characters were examined in specimens that were cleared and stained for bone, and specimens stained for both cartilage and bone. It was noted by Potthoff (1984) that most larval specimens fixed in alcohol disarticulated during the clearing and staining process. Similarly, adult fish specimens that were alcohol fixed disarticulated in the present study. To remedy this, all specimens collected into alcohol were re-fixed in 10% marble chip buffered formalin for at least two days.

Fish specimens were stained for bone using a method modified from Potthoff (1984). Skin pigments were removed by bleaching in a solution of 0.5% H_2O_2 and 1% KOH for between 20 min (small specimens) and 60 min (large specimens). Subsequently, the flesh was cleared by trypsin digestion and bones were stained with alizarin red, based on the method described by Taylor (1967). Trypsin digestion was performed at 28° C in 30% saturated sodium borate solution containing 2% trypsin powder (w:v) until specimens were largely (>60%) transparent. Staining of bones was performed in a solution containing 1% KOH and 1% dye solution (0.6% chloral hydrate, 10% glycerol, 5% glacial acetic acid, 30% saturated alizarin red) for 24 h. Excess alizarin red was removed from the specimen by soaking it in 4% KOH solution for 24 h (Rojo

Table 3.1 Details of specimens cleared and stained for osteological analysis.

Species	# Cleared	Standard length (mm)	Origin	Locality	Latitude	Longitude	Collector
Retropinna tasmanica	1	63	Tasmania	Duck R.	40°52'S	145°08'E	R W. G. White
Prototroctes maraena	1	59	Tasmania	Northwest Bay R.	43°00'S	147°16'W	R. W. G. White
Lepidogalaxias salamandroides	3	30, 26, 25	Western Australia	Chesapeke Rd.	34°38S	116°06W	H. Gill
Lovettia sealii	4	49, 45, 44, 42	Tasmania	Northern Tas.	41°S	147°W	J. Purser
Galaxias auratus	3	65, 63, 58	Tasmania	L. Crescent	42°08'S	147°09'W	R. B. Mawbey
Galaxias brevipinnis	3	82, 70, 59	Tasmania	Snug R.	43°04'S	147°12'W	J. Waters
Galaxias cleaveri	2	81, 60	Tasmania	Allens Ck.	43°03'S	147°53'W	D. Crook
Galaxias fontanus	1	72	Tasmania	Swan R.	41°50'S	148°06'W	J. Waters
Galaxias maculatus	2	92, 81	Tasmania	Sandy Bay Riv.	42°53'S	147°20'W	J. Waters
Galaxias parvus	2	71, 50	Tasmania	48 Ck.	42°58'S	146°21'W	R. W. G. White
Galaxias paucispondylus	2	73, 68	New Zealand	Wilberforce R.	43°02'S	171°10'W	T. Eldon
Galaxias truttaceus	3	72, 69, 68	Tasmania	Allens Ck.	43°03'S	147°53'W	J. Waters
Galaxias vulgaris	2	69, 62	New Zealand	South Island	Unknown	Unknown	C. P Mitchell
Galaxias zebratus	3	42, 37, 34	South Africa	Noetsie R.	34°04'S	23°11'E	P. Skelton
Neochanna apoda	3	68, 62, 59	New Zealand	Hamilton	37°48'S	174°52'W	B. Hicks
Neochanna burrowsius	3	79, 53, 48	New Zealand	Canterbury	44°S	172°W	T. Eldon
Paragalaxias dissimilis	2	40, 36	Tasmania	Great L.	41°55'S	146°42'W	D. Crook
Paragalaxias eleotroides	2	34, 26	Tasmania	Great L.	41°55'S	146°42'W	D. Crook
Paragalaxias mesotes	1	53	Tasmania	Arthurs L.	42°01'S	146°56'W	D. Crook
Galaxiella nigrostriata	3	34, 29, 28	Western Australia	Chesapeke Rd.	34°38S	116°06W	H. Gill
Galaxiella pusilla	3	25, 23, 21	Tasmania	Forester Lodge	41°S	148°W	P. Humphries
Brachygalaxias bullocki	2	45, 43	Chile	L. Rupanco	40°25'S	72°40'E	H. Campos
Brachygalaxias bullocki "gothei"	3	35, 34, 30	Chile	Talca	36°S	72°E	K. Busse
Nesogalaxias neocaledonicus	3	63, 63, 54	New Caledonia	L en Huit	22°S	166°W	C. Pöllabauer

(Rojo 1991). The clearing of stained specimens was completed at 28° C in 30% saturated sodium borate solution containing 2% trypsin powder (w:v). Successfully cleared and stained specimens were then preserved for osteological study. Initially, specimens were placed in a solution containing 30% glycerol and 0.7% KOH for two days. They were transferred to a solution containing 60% glycerol and 0.4% KOH for two days. Finally, specimens were transferred to 100% glycerol and a few thymol crystals were added as a preservative.

Alternatively, some specimens were stained with both alcian blue (for cartilage) and alizarin red (for bone). Fixed specimens were dehydrated in absolute ethanol for two days. Cartilage staining was performed in two 24 h steps: an initial stain solution (70% ethanol, 30% glacial acetic acid, 0.02% alcian blue (w:v)) and a second stain solution (60% ethanol, 40% glacial acetic acid and 0.02% alcian blue (w:v)). The low pH resulting from staining in acetic acid solution impairs the subsequent bleaching process (Potthoff 1984). Consequently, specimens were placed in saturated sodium borate solution for 24 h to neutralise the pH. Specimens were bleached, cleared and stained for bone using the alizarin method as described above.

Cleared and stained specimens were examined with the aid of a *Wild* stereomicroscope and an *Intralux 5000* fibre optic light source. Some drawings were made with the aid of a camera lucida stereomicroscope attachment.

There were several potential problems inherent in the search for osteological characters from which to infer a galaxiine phylogeny. All osteological features exhibit some degree of variation within and between individuals and taxa. Significant variation may occur between the paired bones of an individual specimen. Such differences may include varying levels of ossification, bone shape and proportion, and even the presence or absence of distinct features. For example, a pleural rib may be present on the left but not the right side of a precaudal vertebra; the right hyomandibular may not be the exact mirror image of its left counterpart in the same individual. For these reasons, every attempt was made to examine the features of as many individuals as possible in order to assess levels of variation within and between species. Another problem involves specimens at different levels of development. Within a taxon, many osteological features may vary with specimen size. For example, ethmoid bones may be absent in tiny specimens and appear sequentially with increasing size or age. To counteract this problem, the osteological analysis was restricted to adult or near-adult specimens. In addition, an attempt was made to examine similar sized specimens across a range of taxa. The features of diminutive species such as *Galaxias zebratus* and

members of *Galaxiella* were deliberately compared with similarly small specimens of other taxa such as *Paragalaxias*.

Specimens of 18 galaxiine species were examined for 51 morphological characters. Many of the characters chosen were based on the observations of previous osteological studies. Every specimen was examined for each osteological character, with the exception of one character of Williams (1987, p. 722). He presented observations on the maxilla-mandibular ligament of six galaxiines and several other galaxioid species. I did not examine this ligament. A few morphometric and meristic characters were taken entirely from data published by McDowall (1968, 1970, 1971b) and McDowall and Frankenberg (1981). Species with non-overlapping ranges for morphometric and meristic characters were assigned separate character states; species with overlapping ranges were coded as missing data for that character. For each character, an attempt was made to distinguish between primitive and derived states. The symbol "0" was used to represent the plesiomorphic (ancestral) character state while the apomorphic (derived) state was represented by "1" (or both "1" and "2" for multistate characters). When determining character polarity, it is important to consider a range of outgroup taxa because no single species has the plesiomorphic condition for all characters (Sanford 1990). A taxon generally possesses a combination of ancestral and derived character states. Thus, phylogenetic trees were rooted using a hypothetical ancestral taxon. This taxon was based on the character states observed in outgroup taxa in the current study (*Lovettia sealii*, *Retropinna tasmanica*, *Prototroctes maraena*, *Lepidogalaxias salamandroides*) and on published morphological data for *Aplochiton* and osmerids.

Parsimony analysis of the morphological data matrix was performed with PAUP 3.1 (Swofford 1993). The large amount of computer time required for exhaustive and branch-and-bound search methods was avoided by the choice of the heuristic option, a faster method used to find optimal trees. Heuristic searches were performed with stepwise addition, using the tree bisection-reconnection swapping option, repeated 100 times. All characters were equally weighted. Multistate characters were always treated as unordered, as in Begle (1991), Baldwin and Johnston (1993) and Harold (1993). Separate analyses were performed with binary characters treated as either unordered (Fitch parsimony) or ordered (Wagner parsimony). The relative robustness of various clades was assessed using the bootstrap option, which implements the random resampling strategy of Felsenstein (1985). In this method, heuristic searches were performed on 500 bootstrapped samples of the morphological data matrix. Questions of monophyly were assessed by enforcing monophyletic tree topologies with the

"constraints" option of PAUP. In this method, parsimony analyses were performed to examine the relationship between tree topology and tree length.

3.3 Results

External morphology

1. *Dorsal fin position* Variation in the dorsal fin position of galaxiid species has been well documented by McDowall (1971b, 1973b, c, 1984). In *Aplochiton*, *Lovettia* and *Paragalaxias* the dorsal fin is positioned forward, above the pelvic fins (#1:0). However, in most galaxiines the dorsal fin originates more or less above the vent (#1:1). In *Brachygalaxias* and *Galaxiella* the dorsal fin origin is posterior to the vent and anal fin origin (#1:2). While the dorsal fin of *Galaxias zebratus* is marginally further forward (relative to standard length) than many galaxiines (McDowall 1973b), it is not placed above the pelvic fins as in *Paragalaxias*.

2. *Caudal fin shape* Variation in the form of the galaxiine caudal fin was observed by McDowall (1970, 1971b) and McDowall and Frankenberg (1981). In most species the caudal fin is forked, truncated or emarginate (#2:0). In *Galaxiella*, *Neochanna*, *G. cleaveri*, and *G. parvus* the caudal fin has a rounded margin (#2:1).

3. *Caudal peduncle flanges* McDowall (1978a, 1990) noted that in *Neochanna*, *G. cleaveri* and *Galaxiella* caudal peduncle flanges are strongly developed and virtually confluent with the dorsal and anal fins (#3:1). In most galaxiines the caudal flanges do not extend as far as the dorsal and anal fins (#3:0).

4. *Dark fin margins* As described by McDowall and Frankenberg (1981) and Fulton (1990), the dorsal, anal and pelvic fins of *G. truttaceus* and *G. auratus* have distinctly dark margins (#4:1). Apparently this coloration may also be present in *G. tanycephalus* (Fulton 1990) and *G. postvectis* (McDowall 1990) but is absent from other galaxiines (#4:0).

5. *Longitudinal coloration* Most species lack longitudinal coloration (#5:0). However, members of *Galaxiella* have a bright orange stripe beneath the lateral line (#5:1; McDowall 1978a). Regan (1908) and Scott (1966) noted a similar orange stripe in *Brachygalaxias bullocki*. McDowall (1971b) described an orange patch in large specimens of this species "extending from a little behind the pelvic fins to caudal fin base...". He claimed that the description of this coloration as longitudinal was "hardly appropriate". Subsequently, Berra *et al.* (1995) reported a bright red stripe that extends from under the *pectoral* fin to the caudal fin in large specimens of both sexes. Their

Figure 7 clearly shows that Regan and Scott were correct in their description of coloration as longitudinal.

6. *Submandibular laterosensory pores* There are normally two laterosensory pores present in the lower jaw of galaxiines (#5:0). McDowall and Frankenberg (1981) noted that these submandibular pores are lacking (#5:1) in *Galaxiella*, *Brachygalaxias*, *G. zebratus* and some members of *Paragalaxias*.

7. *Ventral abdominal keel* McDowall (1969) recorded a "fleshy midventral abdominal ridge" (#7:1) in *G. maculatus* and *Brachygalaxias bullocki*. This structure is also present in members of *Galaxiella* but is absent in most galaxiines (#7:0).

8. *Head length* This character is based on the morphometric measurement of head length as a percentage of standard length as published in McDowall (1970, 1971b, 1973b) and McDowall and Frankenberg (1981). Paragalaxiids have long heads, always exceeding 28% of standard length (#8:1). Some other species have head lengths always less than 28% of standard length (#8:2). Species with ranges extending both above and below 28% are coded as missing data for this character (#8:?).

9. *Eye diameter* This character is based on the morphometric measurement of eye diameter as a percentage of head length as published in McDowall (1970, 1971b, 1973b) and McDowall and Frankenberg (1981). *Neochanna*, *G. paucispondylus*, and *G. cleaveri* have small eyes, always less than 20% of head length (#9:2). In contrast, other galaxiines have large eyes, always exceeding 20% of head length (#9:1). Species with ranges extending both above and below 20% are coded as missing data for this character (#9:?).

10. *Prepelvic length* This character is based on the morphometric measurement of prepelvic length as a percentage of standard length as published in McDowall (1970, 1971b, 1973b) and McDowall and Frankenberg (1981). Some galaxiines have a prepelvic length always greater than 54% of standard length (#10:1). Members of *Paragalaxias* have prepelvic lengths consistently less than 54% of standard length (#10:2). Species with ranges extending both above and below 54% are coded as missing data for this character (#10:?).

Endocranium

11. *Median ethmoid ossification* As noted by McDowall (1969) and Frankenberg (1969), many galaxiines may have some or all of a developmental series of distinct median ethmoid bones that includes a supraethmoid, ventral ethmoid and

ethmomyodomal (#11:0). Weitzman (1967) suggested that these three osmerid-type ethmoid ossifications may link galaxioids and osmeroids. In some galaxiines the ethmoid region is very strongly ossified and the three bones are more or less fused into a block (#11:2). Ethmoid fusion has been recorded in *Paragalaxias* (Frankenberg, 1969), *G. paucispondylus* (McDowall 1969) and *G. parvus* (Andrews 1976). *Galaxias cleaveri* and members of the genus *Neochanna* lack median ethmoid ossification (#11:1). Apparently this is also the case in *G. platei* (Weitzman 1967). In the present study an ethmomyodomal and supraethmoid were detected in specimens of *G. zebratus*, reversing the findings of McDowall (1969). My specimens of *Galaxiella* generally lacked median ethmoid ossification; one specimen of *G. nigrostriata* had what appeared to be a weakly ossified supraethmoid. McDowall's (1969 p. 814) claim that "galaxiids with weak ethmoid ossification are...shoal inhabiting species" is inaccurate.

12. *Ethmomyodomal* This is a small conical perichondral bone that is part of the ethmoid series (see character #10), forming the anterior wall of the myodome cavity. It is present in *Lovettia* and most galaxiines (#12:0). However, it is absent (#12:1) from a number of taxa including mudfish, *Nesogalaxias*, *Galaxiella*, *G. maculatus* and *G. auratus*.

13. *Pterosphenoid* Begle (1992) observed that the pterosphenoids of all osmeroids are reduced and do not meet under the midline of the frontals. He considered this to represent a derived condition. Patterson and Johnson (1995) argued that widely separated pterosphenoids are a primitive state in teleosts. In the specimens I examined, the level of pterosphenoid development varied intraspecifically, increasing with size. Most species have weak to moderately developed pterosphenoids with median processes that are well separated from each other (#13:0). However, *Galaxias maculatus* has strongly developed pterosphenoids, the median processes of which are expanded and extend to near the midline (#13:1).

14. *Sphenotic* In most specimens examined, the posterodorsal margin of the sphenotic is overlapped by the anterolateral edge of the parietal (#14:1) as shown in drawings by Williams (1987, p. 543) and McDowall (1969, p. 801). This also appears to be the case in the osmerid *Spirinchus thaleichthys* as illustrated in Weitzman (1967, p. 510). In contrast, the sphenotic is consistently well separated from the parietal margin (#14:0) in retropinnids, *Aplochiton*, *Lovettia* and a few small galaxiine species.

15. *Exoccipitals* The exoccipitals normally extend posteriorly to about the level of the epiotics (#15:0) as shown in Rosen (1974, p. 153). McDowall (1969) noted that the exoccipitals of *G. paucispondylus* and related species form a caudad extension of the

cranium (#15:1). Of the specimens I examined, this caudad extension was also present in *G. parvus* and *N. burrowsius*.

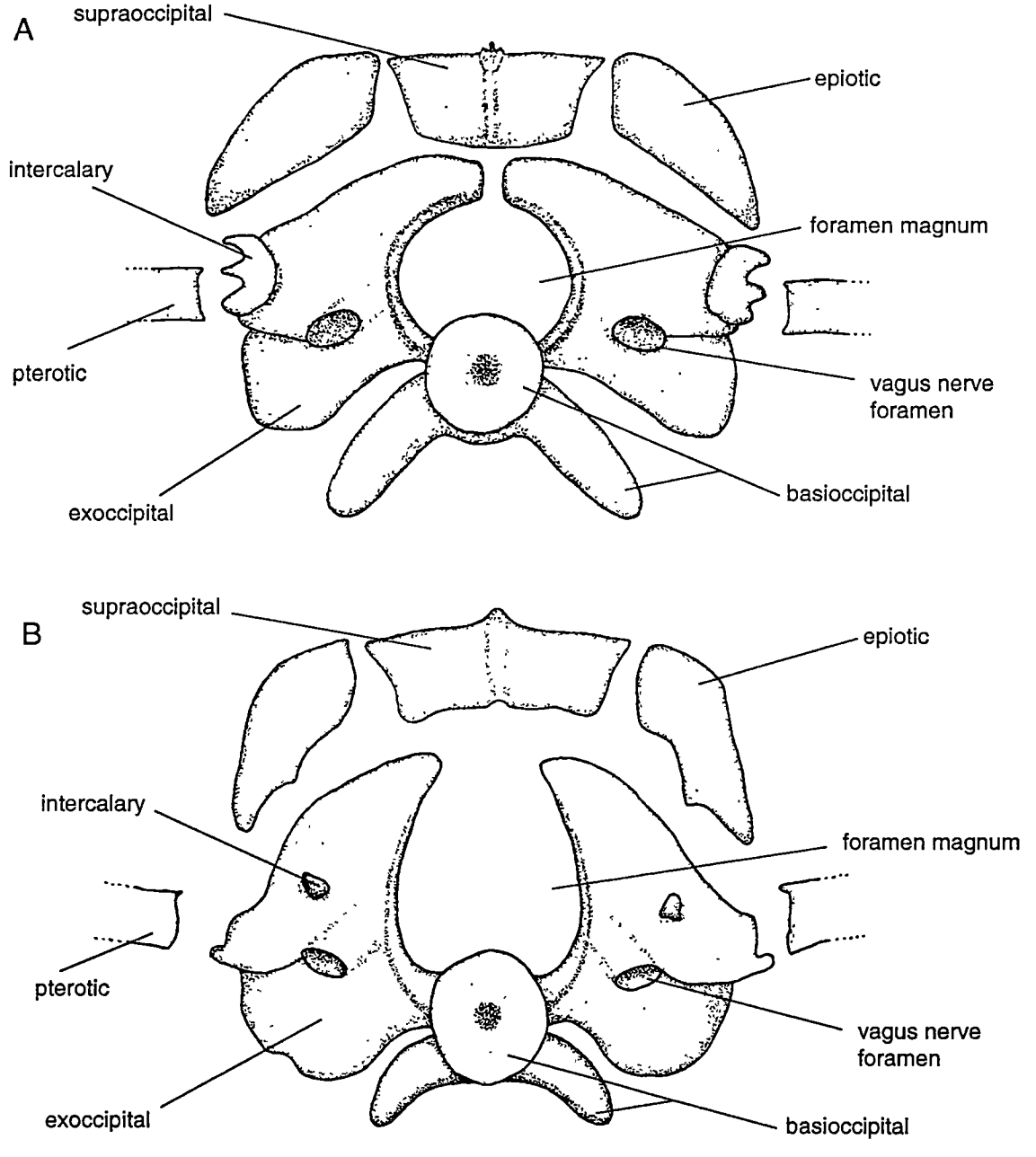

Figure 3.1 Posterior view of the cranium showing position of the intercalary. A. *Paragalaxias dissimilis*; B. *Brachygalaxias bullocki*.

Dermocranium

16. *Intercalar* This small ossification, associated with the posttemporal ligament, was first recognised in galaxiines by Frankenberg (1969). He stated that the intercalar lies either entirely on the exoccipital, or on both the exoccipital and the epiotic. A drawing by McDowall (1969, p. 813) shows the opisthotic (intercalar) on the posterolateral margin of the exoccipital. Of the specimens I examined, most had a well ossified intercalar adjacent to the posterolateral edge of the exoccipital (Fig. 3.1A, #16:0). In

specimens of *Galaxiella*, *Brachygalaxias* and *G. zebratus*, intercalars were small "floating" ossifications placed mesial to the posterolateral margin of the exoccipitals (Fig. 3.1B, #16:1). This was not the case in similarly small specimens of other taxa.

17. *Vomer shaft* Galaxiids are characterised by a toothless vomer with a pointed posterior shaft (Fig. 3.2). Begle (1992, p. 361) incorrectly stated that the posterior shaft is absent from galaxiines, *Lovettia* and *Prototroctes*. In most species, the posterior shaft is of moderate length (#17:0). As noted by Frankenberg (1969) and this study, members of *Paragalaxias* have an unusually long posterior shaft (#16:2). In contrast, Frankenberg (1969) observed that the posterior shaft is reduced or absent in *Brachygalaxias* and *Galaxiella* (#17:1).

18. *Vomer shape* Frankenberg (1969) stated that most galaxiines possess a "T"-shaped vomer (Fig. 3.2A, #18:0). McDowall (1969) noted that members of *Neochanna* have a large "Y"-shaped vomer (Fig. 3.2C and Fig. 3.2D, #18:1) with the two anterior arms projecting forward behind the maxillary heads. Similarly, Andrews (1976) and the current study describe the vomer of *G. cleaveri* as "Y"-shaped, as shown in Fig. 3.2B.

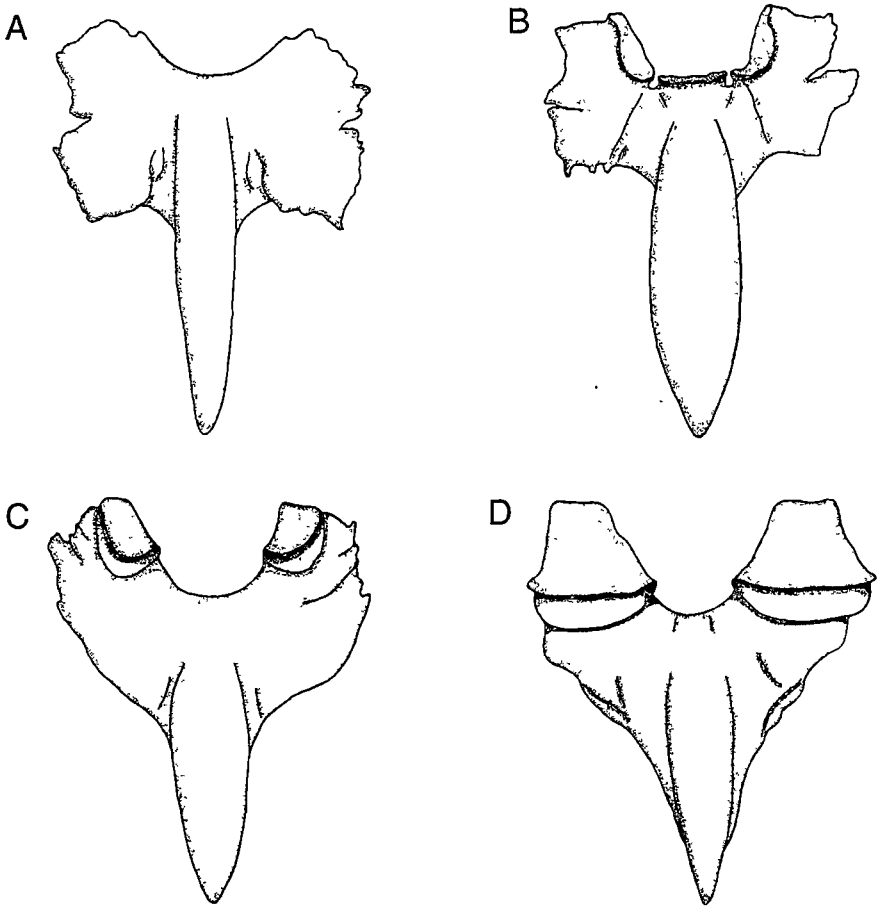

Figure 3.2 Dorsal view of the vomer. A. *Galaxias vulgaris*; B. *Galaxias cleaveri*; C. *Neochanna burrowsius*; D. *Neochanna apoda*.

19. *Upfolding of vomer* McDowall (1969) noted that the vomerine arms of *Neochanna* are strongly upfolded, apparently supporting the premaxillary symphysis (Fig. 3.2C and Fig. 3.2D, #19:1). This is not the case in other galaxiines (#19:0), although the anterior median section of the vomer may be slightly upturned. In *G. cleaveri* both the median section of the vomer and the mesial edges of the arms are upturned (Fig. 3.2B) but this is not nearly as pronounced as the upfolding seen in *Neochanna*.

20. *Parasphenoid* In dorsal view, the parasphenoid appears long and slender in many galaxiine species (#20:0). Occasionally this bone is expanded laterally, appearing much thicker in dorsal view (#20:1). This character state was observed in *Neochanna*, *G. cleaveri*, *G. vulgaris*, *G. brevipinnis*, *G. fontanus*, and *G. paucispondylus*.

21. *Lachrymal flange* The anterior infraorbital (lachrymal) has an anterodorsal flange which abuts the anterior margin of the eye. Frankenberg (1969) described this structure and noted variation in its level of development. In *Aplochiton*, *Lovettia sealii* and most galaxiines this flange is strongly developed (#21:0). Conversely, it is very reduced in *Neochanna*, *G. cleaveri*, *G. zebratus*, and *G. fontanus*. (#21:1).

22. *Antorbital* A small antorbital is present in *Aplochiton*, *Lovettia* and many galaxiines (#22:0). It is particularly well developed in *Nesogalaxias neocaledonicus*. However, antorbitals were absent (#22:1) from several of the species examined.

23. *Supraorbital canal* Frankenberg (1969) noted that the supraorbital canal present on the dorsal surface of each frontal has a lateral wing extending above the orbit. I detected variation in the extent of this lateral wing. In most species examined, the wing of the canal extends to the lateral edge of the frontal above the orbit (Fig. 3.3A, #23:0). In a few species, including *G. cleaveri* and members of *Neochanna*, the canal is strongly developed, curving down below the frontal margin (Fig. 3.3B, #23:1). In these species the lateral wing of the canal largely obscures the pterosphenoid when viewed laterally.

24. *Supraorbital canal* In most galaxiines examined, the lateral wing of the supraorbital canal is largely open dorsally (Fig 3.3A, #24:0). However, in *G. zebratus*, *G. cleaveri* and *N. burrowsius* the lateral wing of the canal is at least half enclosed in bone (Fig 3.3B, #24:1).

25. *Preopercle angle* Williams (1987) noted variation in the angle formed by the dorsal and ventral limbs of the preopercle in galaxiines. In most species the two limbs meet at about a right angle (#25:0). However, the angle is obtuse (#25:1) in *Paragalaxias*, *G. parvus*, *G. paucispondylus* and *Brachygalaxias bullocki*.

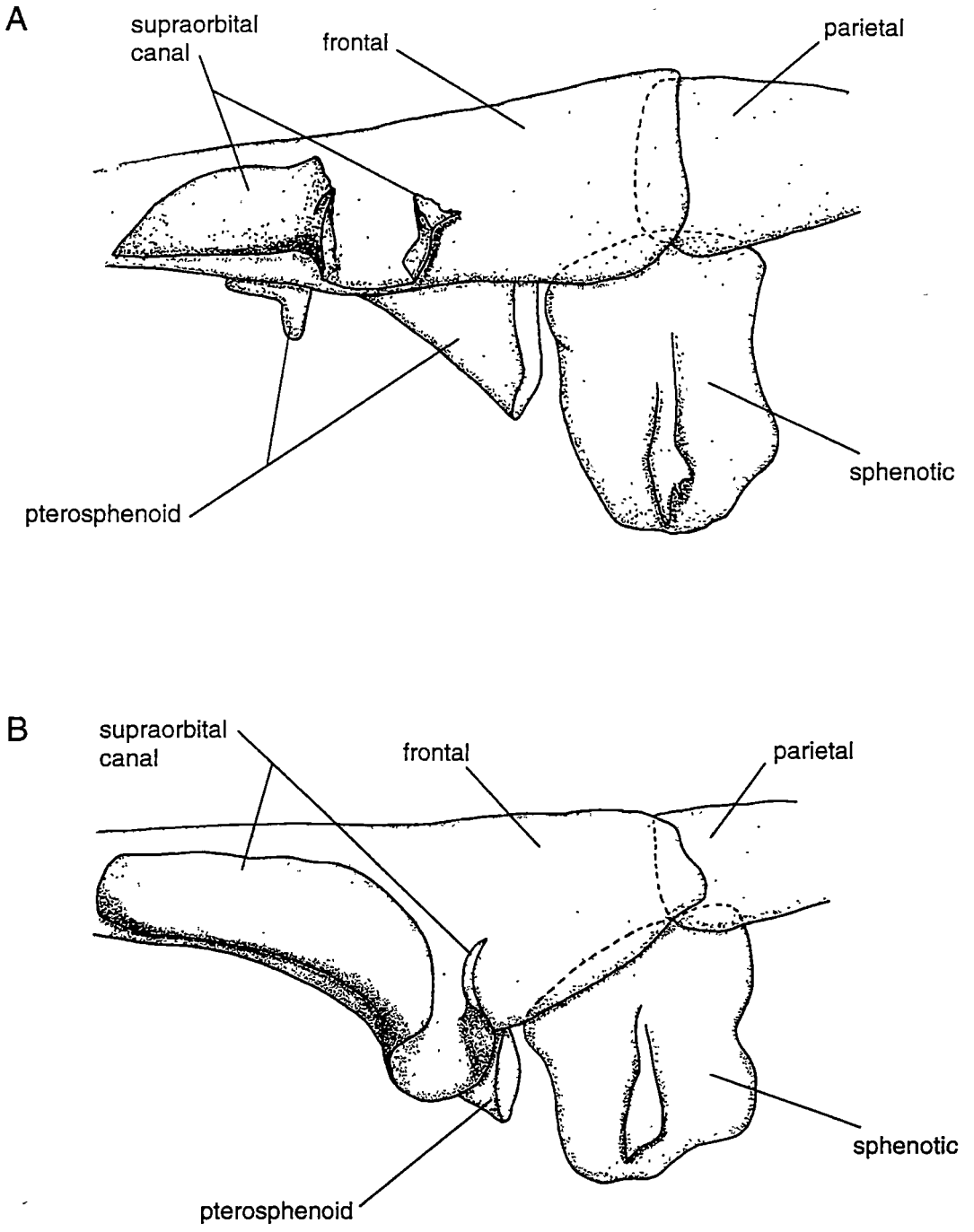

Figure 3.3 Lateral view of the left frontal region showing the supraorbital canal. A. *Galaxias auratus*; B. *Galaxias cleaveri*.

26. *Preopercular dorsal limb* Frankenberg (1969) and Williams (1987) described variation in the length of the preopercular dorsal limb. In some galaxiines (and other galaxioids) the dorsal limb is relatively short, not extending above the top of the opercular arm (#26:0). Conversely, the preopercle extends above the opercular arm of the hyomandibular of most species (#26:1).

27. *Preopercle anterior lamina* Williams (1987) stated that "the anterior lamina had the same basic shape" in his galaxiine specimens but noted variation in the size of the lamina. Most galaxiines have a moderately-sized or small anterior lamina (#27:0). However, the preopercles of *G. brevipinnis*, *G. fontanus*, *G. vulgaris* and *Nesogalaxias neocaledonicus* have very large anterior laminae (#27:1).

Splanchnocranium

28. *Ascending process of premaxilla* McDowall (1969) observed that in species of *Neochanna* "the ascending processes of the premaxillae are pushed posteriorly...to nearly meet the tips of the frontals", as shown in his diagram of *N. diversus* (p. 813). This was the case in my specimens of *N. burrowsius* and *N. apoda* with the ascending processes approaching but not contacting the frontals. Similarly, Andrews (1973) described the ascending process in *G. cleaveri* as "expanded lateroposteriorly" and overlying the vomer. I found that the ascending process of the premaxilla in *G. cleaveri*, *G. zebratus*, and members of *Neochanna* extends posteriorly to between the nasals (#28:1). In other specimens examined, the ascending processes do not extend between the nasals (#28:0).

29. *Alveolar process of premaxilla* As noted by McDowall (1969), a long alveolar process of the premaxilla totally excludes the maxilla from the gape in some galaxioids. This apparently primitive condition is present in *Stokellia*, *Prototroctes*, *Lovettia*, *Aplochiton* and *Brachygalaxias bullocki* (#29:0). In all other galaxiines, the maxilla is only partially excluded from the gape (#29:1).

30. *Maxilla flange* The galaxiine maxilla is long, slender and toothless. In most species, the maxilla shaft is smooth and gently curving (Fig. 3.4A, #30:0). There is a distinctive dorsoposterior flange extending from the maxilla about halfway along its length in *G. paucispondylus* and *G. brevipinnis* (Fig 3.4B and Fig. 3.4C, #30:1). This flange gives the maxilla a "bent" appearance and may be similar to the structure present in *Plecoglossus* and the fossil osmerid *Speirsaenigma lindoei* (Wilson and Williams 1991).

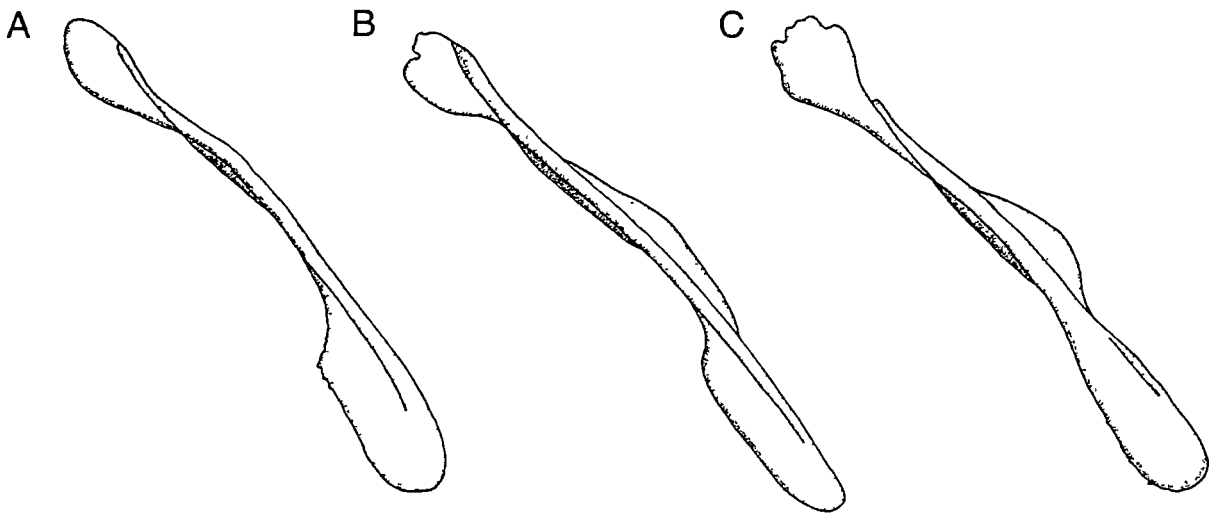

Figure 3.4 Dorsolateral view of the left maxilla. A. *Galaxias truttaceus*; B. *Galaxias brevipinnis*; C. *Galaxias paucispondylus*.

31. *Maxilla expansion* The posterior portion of the maxilla is expanded dorsoventrally and rounded to give an "oar-like" appearance (Fig. 3.4). In most species this expanded section represents less than half the length of the maxilla (#31:0). However, in members of the genus *Galaxiella*, the expanded section continues for at least half the length of the maxilla (#31:1).

32. *Maxilla-mandibular ligament* Williams (1987) produced a morphocline postulating evolutionary changes in the maxilla-mandibular ligament of galaxioids. He noted that the anterior section of this ligament, which joins the maxilla, is long and slender (#32:0) in *Aplochiton*, *Lovettia sealii* and *G. maculatus*. Conversely, in *G. paucispondylus*, *G. brevipinnis*, *G. fasciatus*, *Brachygalaxias* and *Neochanna* the anterior section is considerably shorter and thickened (#32:1). Other species were not examined for this character.

33. *Palatine lamina* The galaxiine palatine lacks teeth and a dermopalatine (Williams 1987). Most species have distinctive lateral and/or medial dermal laminae (#33:0). I found both of these laminae to be absent from specimens of *G. truttaceus* and *G. auratus* (#33:1). The palatine of *Galaxiella pusilla* was very weakly ossified; no laminae were observed in my specimens of *G. nigrostriata*.

34. *Palatine spur* A posterolateral splint of bone (#34:1) often extends from the margin of the palatine towards the quadrate (Frankenberg 1969; McDowall 1969; Andrews 1976; Williams 1987). It is long and particularly well developed in *G. cleaveri* and

members of *Neochanna* (#34:2). Conversely, the palatine spur is absent in *Aplochiton*, *Lovettia* and several galaxiines (#34:0).

35. *Mesopterygoidal teeth* Most galaxiines have several well developed teeth in a single row on the mesioventral surface of the the mesopterygoid (#35:0). In *G. cleaveri* and members of *Neochanna*, mesopterygoidal teeth are normally few in number or absent (#35:1). Stokell (1945) noted the presence of a few small teeth in some specimens of *N. burrowsius*. McDowall (1970) found a single tooth on each mesopterygoid in one specimen of *N. diversus*. Similarly, Andrews (1976) found that *G. cleaveri* may have one, two or no mesopterygoidal teeth.

36. *Metapterygoid* Frankenberg (1969) noted that the metapterygoid is usually well developed, contacting both the hyomandibular and the symplectic (#36:0). However, in *Galaxiella* the metapterygoid is reduced and does not reach the hyomandibular (#36:1). Williams (1987) observed a reduced metapterygoid in *G. paucispondylus*, but this was not the case in my specimens of this species. I support Williams' suggestion that this discrepancy is due to the small size of his *G. paucispondylus* specimen (56 mm).

37. *Hyomandibular ventral arm* The dimensions of the hyomandibular's ventral arm vary. In most species the arm is longer than it is wide (Fig. 3.5A, #37:0). In contrast, the width of the ventral arm exceeds its length (Fig. 3.5B, #37:1) in *Galaxiella*, *Neochanna apoda*, *N. burrowsius* and *G. cleaveri.*

38. *Hyomandibular posterodorsal lamina* Williams (1987) described the hyomandibular of *Aplochiton* and galaxiines as possessing a distinct posterodorsal lamina (see diagrams on his p. 541 and 544). I found this to be generally the case (Fig. 3.5, #38:0), but the posterodorsal lamina was absent (#38:1) in *G. zebratus, Galaxiella* and *Brachygalaxias bullocki.*

39. *Third hypobranchial* The hypobranchials are small bones in the gill apparatus which are placed mesioventral to the ceratobranchials and laterodorsal to the basibranchials. In most galaxiines examined, the third hypobranchial has a moderately developed process (Fig. 3.6A, #39:0) that extends anteromedially towards the third basibranchial. However, in members of *Neochanna* this distinct process extends medially, perpendicular to the body of the third hypobranchial (Fig 3.6B, #39:1).

Pectoral girdle

40. *Postcleithrum* The pectoral girdle of *Lovettia sealii* and most galaxiines contains a single postcleithrum (#40:0). McDowall (1969) observed that the postcleithrum is

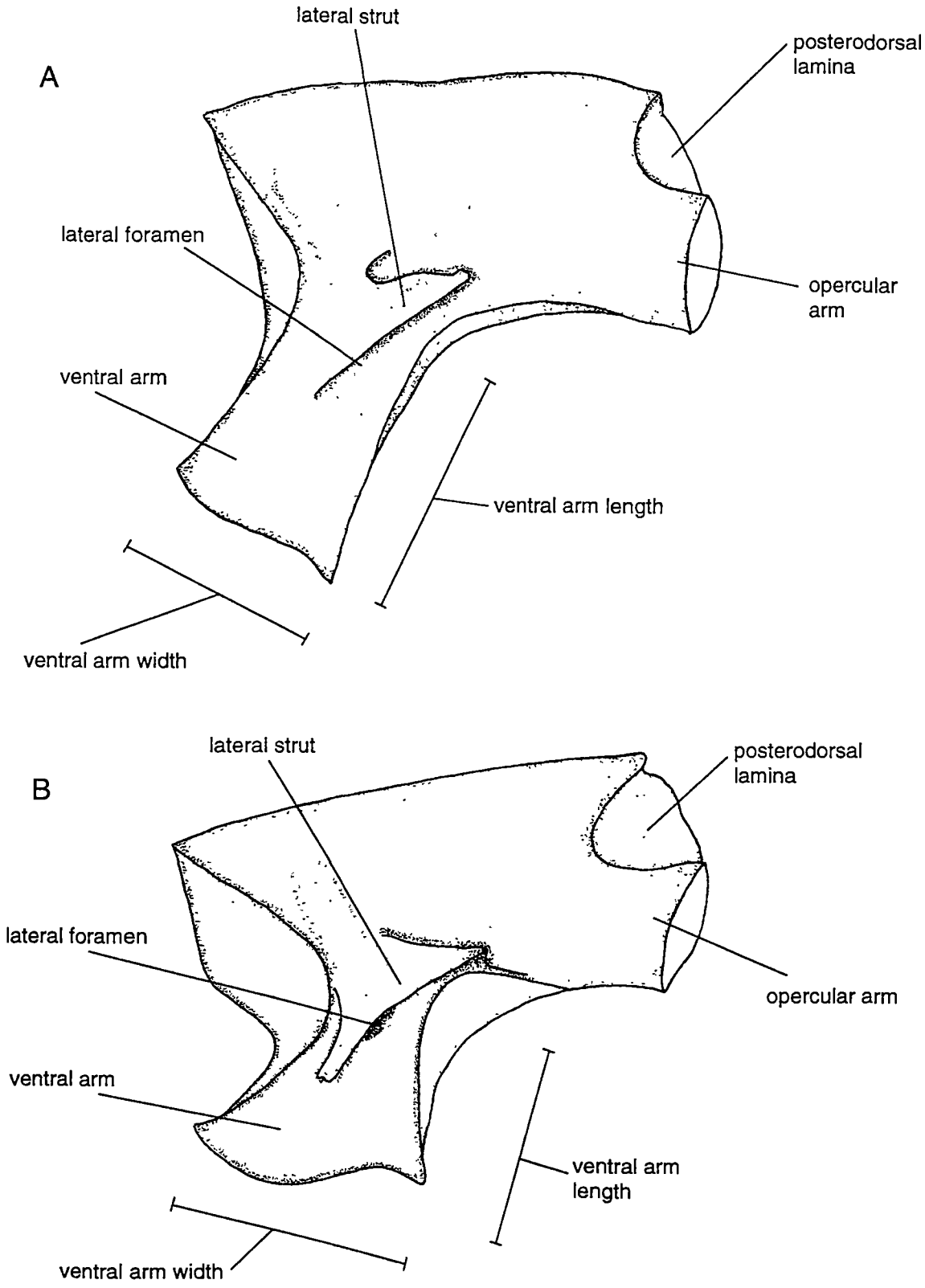

Figure 3.5 Lateral view of the left hyomandibular. A. *Galaxias truttaceus*; B. *Neochanna apoda*.

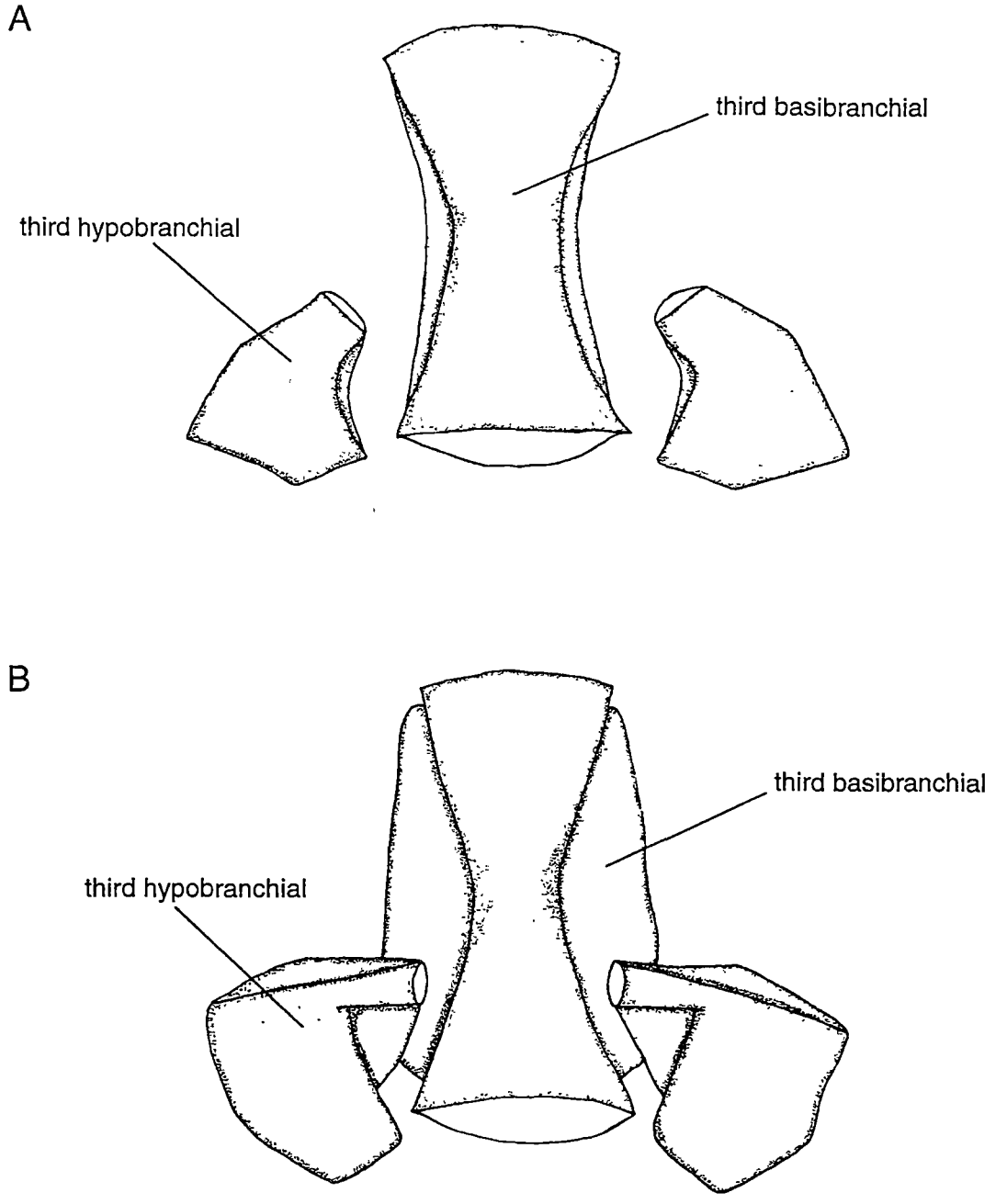

Figure 3.6 Ventral view of the third hypobranchials and third basibranchial. A. *Galaxias zebratus*; B. *Neochanna burrowsius*.

absent (#40:1) from species including *Aplochiton*, *G. paucispondylus* and *N. neocaledonicus*. According to Frankenberg (1969), *G. olidus* may have two postcleithra.

41. *Scapular foramen* The scapular forms part of the primary pectoral girdle. In most galaxioids there is a foramen in the the dorsal part of the scapular, near its articulation with the cleithrum (#41:0). The scapular foramen is not present in *Aplochiton*, *Lovettia*, and *G. zebratus* (#41:1).

42. *Coracoid cartilage* *Aplochiton zebra* and *Lovettia sealii* both possess a finger-like posterior projection of their coracoid cartilage. This cartilaginous finger extends alongside the fourth radial of the pectoral girdle, as shown in McDowall (1969, p. 807). Although normally absent (#42:0), a similar extension was observed in the pectoral girdles of some galaxiine species (#42:1).

43. *Coracoid orientation* The galaxiines exhibit variation in the angle of the coracoids. In many species, the coracoid face has a lateroventral disposition (#43:0). The coracoid has a lateral orientation (#43:1) in mudfish, *Galaxiella*, *Brachygalaxias bullocki* and *G. zebratus*. In *G. brevipinnis* and *G. vulgaris* the face of the coracoid has a ventral orientation (#43:2), corresponding to the ventral orientation of the pectoral fin lamina in these species.

Pelvic girdle

44. *Pelvic rays* McDowall (1969, 1970, 1971b) and McDowall and Frankenberg (1981) observed that pelvic fin ray numbers vary between species. There are typically seven pelvic rays (#44:0) but this number is reduced to six or less (#44:1) in some taxa including *Paragalaxias*, *Galaxiella* and *Neochanna*.

45. *Pelvic bone length* The pelvic bone typically represents less than 5% of the standard length of cleared galaxiine specimens (#45:0). However, the pelvic bone is relatively large in *G. brevipinnis*, *G. vulgaris*, *G. paucispondylus* and *N. neocaledonicus*. In these species, the bone represents more than 5% of the standard length (#45:1). It should be remembered that the clearing process reduces the standard length.

Axial skeleton

46. *Pleural ribs* Patterson and Johnson (1995) observed that the origin of the first pleural rib on the third vertebra (V3) is "remarkably constant in lower teleosts". However, they stated that the osmeroids are exceptions with pleural ribs originating on V2 (#46:0). Frankenberg (1969) noted that pleural ribs are associated with, but do not

contact, V1 in *Paragalaxias* (#46:1). I found this to be the case in all my specimens of *Paragalaxias*. Elsewhere in the teleosts, this condition has been reported only in *Paralepis*, *Alepisaurus*, and *Omosudis*. In these taxa the ribs are fused to the centra of V1 and V2 (Patterson and Johnson 1995).

47. *Epineurals* Most galaxiines have one pair of epineurals associated with the first vertebra (#47:0). Frankenberg (1969) and Andrews (1976) both noted that the first vertebra of *G. cleaveri* has two pairs of interneurals (#47:1). I found two pairs in specimens of *G. truttaceus*, *G. auratus* and *Neochanna apoda*. In contrast, the first vertebrae of *Brachygalaxias* and *Lovettia* lack epineurals (#47:2).

48. *Haemal and neural spines* The caudal neural and haemal spines are usually strongly compressed (#48:0). However, McDowall and Frankenberg (1981) noted that the caudal spines of *Galaxiella* are slender spikes and not laterally flattened (#48:1). Similarly, I found this to be the case in *G. zebratus*.

49. *Supraneural series* Frankenberg (1969) noted the presence of a supraneural series in the predorsal region of galaxiines. The series usually extends nearly to (sometimes overlapping) the pterygiophores of the first dorsal fin (#49:0). However Frankenberg observed that the supraneural series is reduced in some species (#49:1). In *Galaxiella nigrostriata* the series never extends more than two-thirds of the distance to the dorsal fin; in *G. pusilla* and *Brachygalaxias bullocki* the series may be absent entirely.

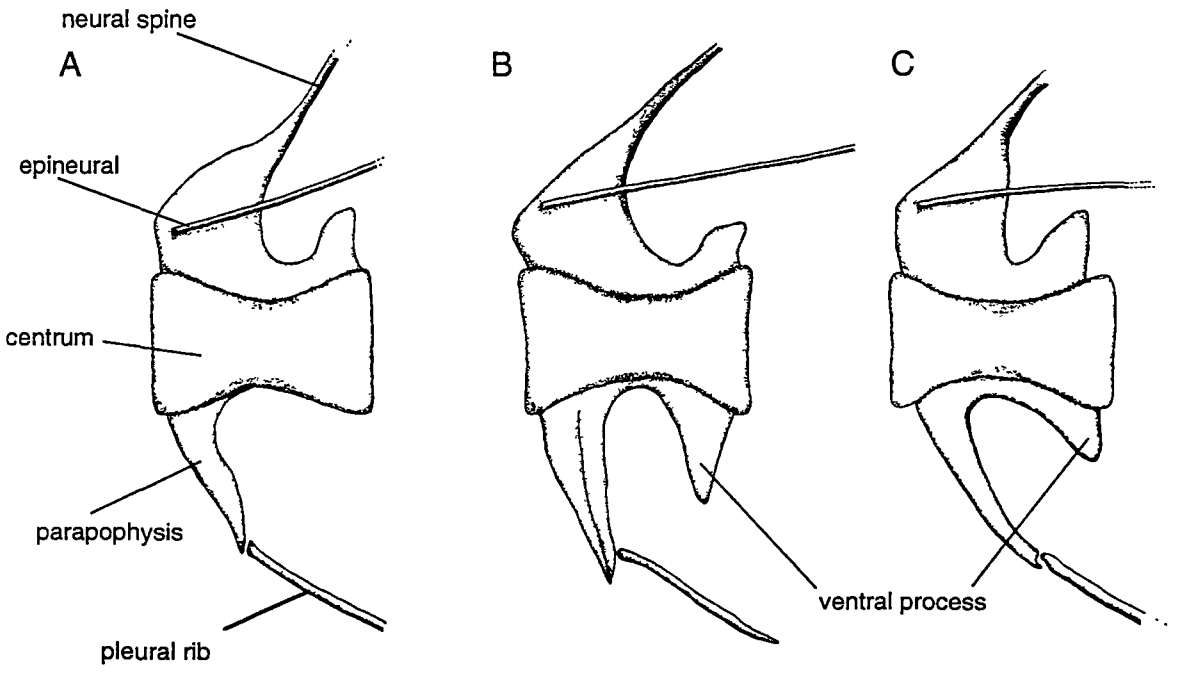

Figure 3.7 Lateral view of posterior precaudal centrum. A. *Galaxias maculatus*; B. *Nesogalaxias neocaledonicus*; C. *Galaxias vulgaris*.

50. *Ventral processes of centra* Frankenberg (1969) described "processes extending ventrally from the posterior part of the centrum" on the posterior precaudal vertebrae and anterior caudal vertebrae of *Nesogalaxias*. The current study supports this finding (Fig. 3.7B, #50:1). Similar processes were observed in *G. vulgaris* (Fig. 3.7C) but are lacking in other species (Fig. 3.7A, #50:0).

51. *Caudal skeleton* The galaxiine caudal skeleton is characterised by five hypurals. In most species the hypurals are distinct (#51:0). In *Brachygalaxias*, *Galaxiella* and *G. zebratus* the hypurals are more or less fused (#51:1), as illustrated in McDowall (1973c, p. 194).

The aforementioned 51 characters were assembled to form a morphological data matrix (Table 3.2). Maximum parsimony analysis of this matrix with all characters unordered produced 14 most parsimonious (mp) trees, each 104 steps in length (consistency index, 0.538; retention index, 0.694). Reanalysis with all characters ordered (except multistate characters) found the same 14 mp trees. Strict consensus analysis by PAUP yielded a consensus cladogram (Fig. 3.8), showing the phylogenetic relationships common to all 14 mp trees.

Three groupings were present in most (but not all) of the mp trees and are shown in the majority rule consensus tree (Fig. 3.9). *Galaxias parvus* was grouped with *Paragalaxias* in all but two trees, *G. brevipinnis* and *G. vulgaris* formed a clade in eight trees, and the *G. brevipinnis* clade (five species) was separated from the large unresolved polycotomy in eight of the 14 trees. Bootstrapping (500 replicates, Fig. 3.9) provided statistical support for several groups. Five widely accepted groups received support: *Galaxiella*, *Paragalaxias*, *Neochanna*, *Galaxias truttaceus-G. auratus*, and *G. brevipinnis-G. vulgaris*. In addition, *G. cleaveri* was grouped with *Neochanna* at a bootstrap level of 100% and there was strong support for the clade [*G. zebratus*, [*Brachygalaxias*, *Galaxiella*]].

The morphological analysis provided support for the non-monophyly of the genus *Galaxias*. As stated above, there is evidence that *G. cleaveri* and *G. zebratus* are primitive members of other galaxiine clades. It was found that 19 additional steps (123) were required for the monophyly of this genus. However, with the removal of *G. cleaveri* and *G. zebratus* from *Galaxias*, only four additional steps were required.

3.4 Discussion

Some variable osteological features discussed by previous workers were found to be highly homoplasious or uninformative and were excluded from the current analysis.

Table 3.2 Morphological data matrix of 51 characters for 18 galaxiine species and a hypothetical outgroup taxon.

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51
Hypothetical ancestor	0	0	0	0	0	0	0	?	?	?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Galaxias auratus	1	0	0	1	0	0	0	?	?	?	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	?	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
Galaxias truttaceus	1	0	0	1	0	0	0	?	?	?	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	?	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
Galaxias maculatus	1	0	0	0	0	0	1	2	?	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
Galaxias fontanus	1	0	0	0	0	0	0	2	?	1	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	1	0	1	0	0	?	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Galaxias brevipinnis	1	0	0	0	0	0	0	?	?	?	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	1	0	1	1	1	0	0	0	0	0	0	1	0	2	0	1	0	0	0	0	0
Galaxias vulgaris	1	0	0	0	0	0	0	?	?	?	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	?	1	1	0	0	0	0	0	0	1	0	2	0	1	0	0	0	1	0
Galaxias paucispondylus	1	0	0	0	0	0	0	2	2	?	2	0	0	1	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	1	0	1	1	0	0	0	0	0	0	1	1	?	0	0	1	0	0	0	1	0
Galaxias parvus	1	1	0	0	0	0	0	2	?	?	2	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	?	1	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0
Galaxias zebratus	1	0	0	0	0	1	0	2	?	?	0	0	0	0	0	1	0	0	0	0	1	1	1	1	0	1	0	1	1	0	0	?	1	0	0	0	0	1	0	0	0	1	0	1	0	0	0	1	0	0	1
Galaxias cleaveri	1	1	1	0	0	0	0	2	2	?	1	1	0	1	0	0	0	1	0	1	1	1	1	1	0	1	0	1	1	0	0	?	1	2	1	0	1	0	0	0	1	?	1	1	0	0	1	0	0	0	0
Neochanna burrowsius	1	1	1	0	0	0	0	2	2	?	1	1	0	1	1	0	0	1	1	1	1	1	1	1	0	1	0	1	1	0	0	?	1	2	1	0	1	0	1	0	1	0	1	1	0	0	0	0	0	0	0
Neochanna apoda	1	1	1	0	0	0	0	2	2	?	1	1	0	1	0	0	0	1	1	1	1	1	1	0	0	1	0	1	1	0	0	1	1	2	1	0	1	0	1	0	1	1	1	1	0	0	1	0	0	0	0
Paragalaxias mesotes	0	0	0	0	0	1	0	1	?	2	2	0	0	1	0	0	2	0	0	0	0	1	0	0	1	0	0	0	1	0	0	?	1	1	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0
Paragalaxias dissimilis	0	0	0	0	0	0	0	1	?	2	2	0	0	1	0	0	2	0	0	0	0	1	0	0	1	0	0	0	1	0	0	?	1	1	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0
Galaxiella nigrostriata	2	1	1	0	1	1	1	2	1	1	?	1	0	0	0	1	1	0	0	0	0	?	0	0	0	1	0	0	1	0	1	?	0	?	0	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	1
Galaxiella pusilla	2	1	1	0	1	1	1	2	1	1	1	1	0	0	0	1	1	0	0	0	0	?	0	0	0	1	0	0	1	0	1	?	?	?	0	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	1
Brachygalaxias bullocki	2	0	0	0	1	1	1	?	1	?	0	0	0	0	0	1	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	?	1	0	0	0	0	1	0	0	1	1	1	1	0	0	2	0	1	0	1
Nesogalaxias neocaledonicus	1	0	0	0	0	0	0	1	?	?	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	?	1	1	0	0	0	0	0	1	1	?	0	0	1	0	0	0	0	1	0

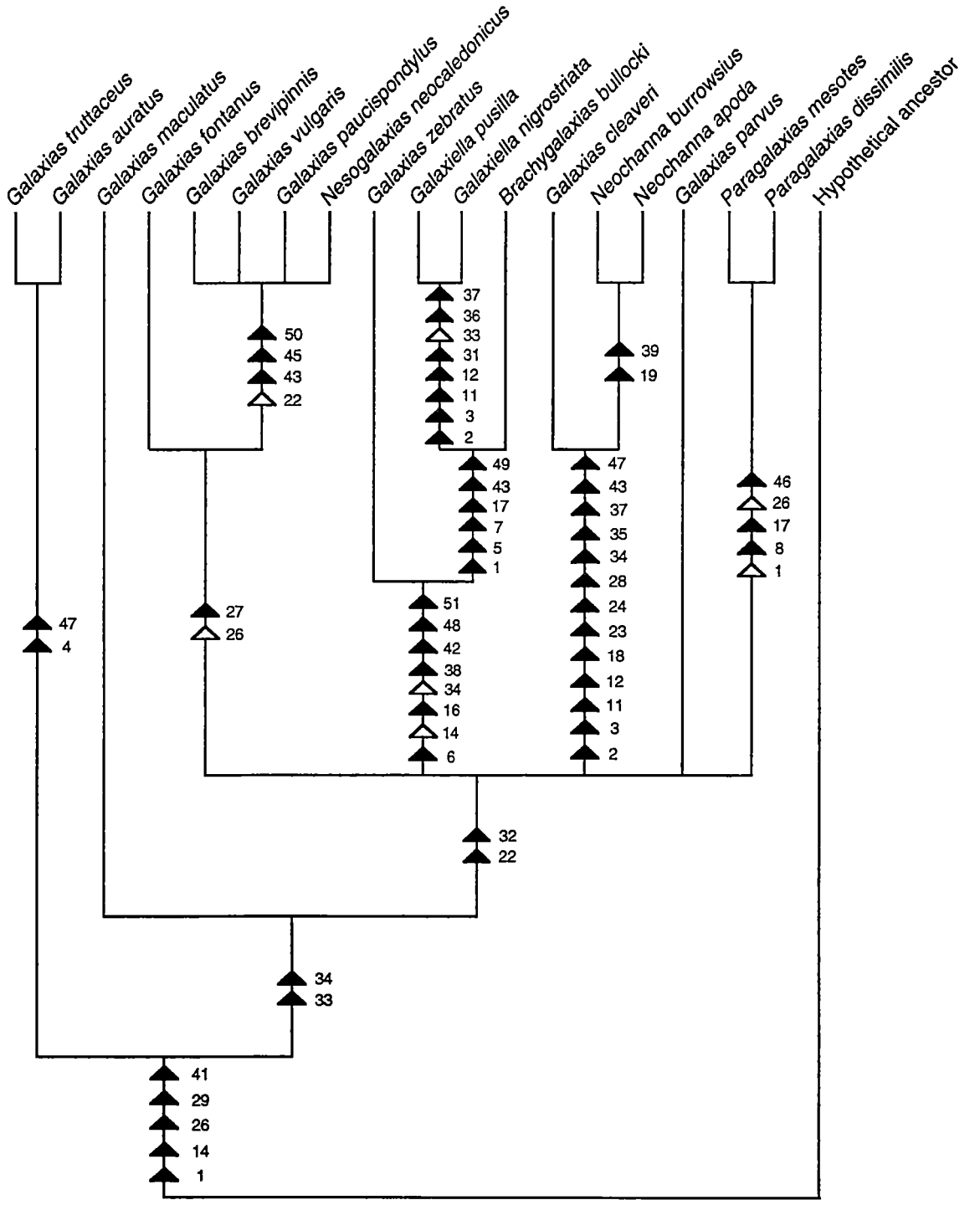

Figure 3.8 Strict consensus cladogram of 14 mp trees (104 steps) from 51 morphological characters (Table 3.2). Autapomorphies of terminal taxa and character changes confounded by the strict consensus are omitted. Closed triangles represent changes to apomorphic states. Open triangles indicate reversals to the plesiomorphic condition.

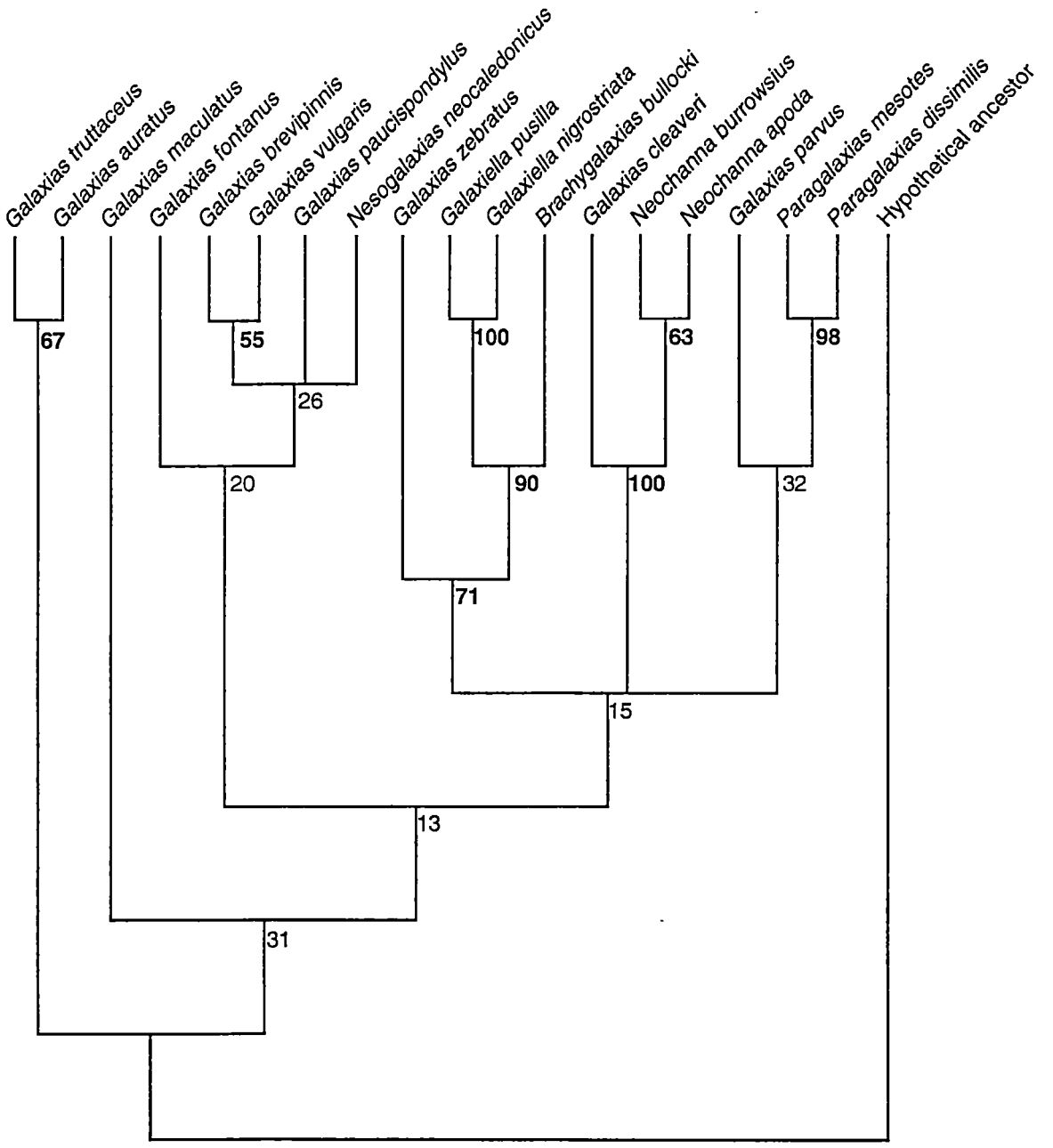

Figure 3.9 Majority rule consensus cladogram of 14 mp trees (104 steps) from 51 morphological characters (Table 3.2). Values at the nodes represent bootstrap percentages based on 500 resamplings. Scores over 50 are shown in bold.

Basioccipital pegs as described by McDowall (1969) were absent from all the specimens I examined. Frankenberg (1969, p. 23) described variation in the position of the hyomandibular lateral foramen relative to the lateral strut. He noted that the foramen may open posterior or entirely anterior to the strut. I found the latter case to be true in *Galaxias cleaveri*, while the the foramen was posterior to the anterior edge of the strut in all other galaxiines. Williams (1987, p. 164) observed that in *Neochanna apoda* the foramen "is located directly medioventral to the ventralmost edge of the lateral strut". This was not the case in my specimens of *Neochanna*. Rather, I found that the foramen was located slightly posterodorsal and medial to the anterior end of the strut (see Fig 3.5B). There is variation in the position of insertion of the primary pectoral girdle, as noted by Frankenberg (1969, p. 25). He recorded the greatest differences between the "closely related species" *Galaxias truttaceus* and *G. brevipinnis*. I observed that in some species the coracoids are attached to cleithrum symphisis whereas in other species they are inserted on the mesial sides of the cleithra, well posterior to the symphysis. However, there is substantial intraspecific variation: I found both states to be expressed within *Brachygalaxias bullocki*. McDowall (1969, p. 811) suggested that galaxiines typically have one caudal centrum but noted the presence of two centra in the caudal skeleton of *Brachygalaxias bullocki*. McDowall (1973c) noted that two distinct urostylar centra are present in many small galaxiine specimens, becoming fused only in larger specimens. My findings support this observation.

Some morphological features may be relatively plastic, making them likely candidates for parallelisms. For example, parallel shortening of the hyomandibular ventral arm has apparently occurred in *Galaxiella* and mudfishes. The galaxiine caudal fin appears to have become rounded in three independent events: in mudfishes, *Galaxiella* and *Galaxias parvus*. The loss of the scapular foramen in *G. zebratus*, *Aplochiton* and *Lovettia* may represent two or three parallel events. In addition, variable characters may be prone to reversals. The position of the galaxiine dorsal fin, while generally conserved, may be occasionally subject to rapid modification. The forward position in *Paragalaxias* appears to represent a return (reversal) to the plesiomorphic condition rather than evidence that this group is ancestral to other galaxiines. Similarly, the unusually long alveolar process of the premaxilla in *Brachygalaxias* is probably a secondarily derived characteristic. In many respects *Brachygalaxias* (longitudinal coloration, large eyes, posterior dorsal fin) and *Paragalaxias* (long head, reduced prepelvic length, long vomerine shaft, fused ethmoid bones) are highly derived taxa. Parsimony analysis of a large morphological data set enables such reversals and parallelisms to be pinpointed.

Conversely, some particularly distinct characteristics are probably less likely to be subject to reversals. Weitzman (1967) noted the presence of "three osmerid types of ethmoid ossifications" in galaxiines. These bones add support to the widely accepted common ancestry of galaxioids and osmerids. However, ethmoid ossifications are absent from all retropinnids, *Aplochiton* and a few galaxiine species. The fact that several close outgroup taxa lack these ossifications does not require the galaxiine ethmoid bones to be interpreted as reversals. It is clear that the presence of an osmerid type supraethmoid, ventral ethmoid and ethmomyodomal is primitive for galaxiines. The loss of these bones has occurred at least three times within the galaxioids and is a secondarily derived state. That is not to say that this condition has no phylogenetic utility for the galaxiines. Rather, it is diagnostic for the mudfish clade.

The origin of well developed epipleural ribs in galaxiines is apparently problematic. McDowall (1969, 1971b, 1978a) and McDowall and Fulton (1978) reported the presence of epipleural ribs in *Galaxias*, *Brachygalaxias*, *Galaxiella* and *Paragalaxias*. Furthermore, McDowall (1969) stated that epipleurals are absent in *Nesogalaxias neocaledonicus*, and that *Neochanna* lacks both epineurals and epipleurals. McDowall (1970) claimed that all three species of *Neochanna* lack epipleurals, in direct contrast to all other New Zealand galaxiines. This was a major consideration in his inclusion of these mudfish in a separate genus. I found epineurals to be invariably present in *Neochanna apoda* and *N. burrowsius*. While my specimens of *N. apoda* lacked epipleural ribs, two specimens of *N. burrowius* had well developed series of epipleurals while a smaller third specimen had none. Of my *N. neocaledonicus* specimens, all three had a reduced series of two or three pairs of epipleurals posterior to the pelvic fins. Similarly, Frankenberg (1969, p. 31) reported a reduced series of "as few as six" pairs in this species.

Begle (1991, 1992) placed the osmeroids as a sister group to the argentinoids, which have well developed epipleurals. However, Patterson and Johnson (1995) argued that osmeroids are immediately related to salmonoids because both groups "share the absence of ossified epipleurals". They noted that members of *Galaxias* and *Paragalaxias* are exceptions in that they have epipleurals. They were apparently unaware of the reported presence of epipleurals also in *Brachygalaxias* and *Galaxiella* (see above). The observations of the present study indicate that epipleurals are present in all six galaxiine genera, and in a vast majority of galaxiine species. Indeed, epipleurals are reduced or absent in only a few highly derived species. Patterson and Johnson (1995) claimed that within the osmerids, epipleurals are present only in *Spirinchus*. This directly conflicts with the finding of Wilson and Williams (1991, p. 446) that epipleural bones are present in all osmerid genera with the sole exception of *Plecoglossus*. It appears that,

with respect to osmeroid epipleurals, the conclusions of Patterson and Johnson (1995) are flawed, owing to their examination of only a few osmeroid specimens and their inadequate search of the literature on this group.

It is true that epipleurals are absent from a few osmeroid taxa. However, Patterson and Johnson (1995) claimed that epipleural absence is primitive for osmeroids and that the presence of these bones represents a reversal. When faced with a choice between multiple gains and multiple losses, they favoured the former. This is surprising given that, elsewhere, they refrained from such a choice. For example, in seeking to explain the disjunct distribution of ossification in teleostean epipleural ligaments (p. 13) they were "unable to choose between the alternatives" of synapomorphous (multiple losses) and homoplastic (multiple gains) hypotheses. With respect to the presence of epipleurals in osmeroids, I believe that the evidence for multiple losses is compelling. These fishes are characterised by reductive evolution. Indeed, Patterson and Johnson (1995) reported that even the epipleural ligaments have been lost in at least *Stokellia*, *Retropinna* and *Lepidogalaxias*. There is evidence to suggest that the proto-salmonoid lacked epipleural ossifications (Patterson and Johnson 1995). In contrast, the occurrence of epipleurals in 13 osmeroid genera, and widely in the lower teleosts, suggests that their presence in galaxiines is a primitive condition rather than a secondarily derived state.

Although not included in the current morphological analysis, the phylogenetic distributions of some galaxioid features are ambiguous and deserve attention. For example, a peculiar cucumber odour is present in fresh specimens of *Retropinna*, *Stokellia*, *Prototroctes*, and the osmerids *Thaleichthys* and *Osmerus* (McDowall 1979; McDowall *et al* 1993). It is caused by the presence of *trans*-2-*cis*-6-nonadienal in the skin (Berra *et al.* 1982). There are two possible hypotheses for the presence of this feature in both osmerids and galaxioids. Begle (1991) suggested that parallel evolution of cucumber odour has occurred in separate stocks. This view implies that cucumber odour in galaxioids and smelts is analogous rather than homologous, although it could be termed *homoiologous* because this parallelism involves close relatives (Hennig 1966). Alternatively, cucumber odour may have been present in the ancestral osmeroid and subsequently lost in most lineages. The former hypothesis (analogy/homoiology) is supported by possibilities such as parallel selection on homologous features and the re-expression of ancestral structural gene complexes. Parallelisms are more likely to occur in closely related taxa (Sluys 1989). For example, it is thought that endothermy has arisen independently in three scombroid lineages (Block *et al.* 1993). Conversely, the latter hypothesis (homology) is supported by the fact that, where present, the odour is chemically homologous. Further evidence for a primitive origin includes the presence

of a similar odour in some argentinids (Muus and Dahlstrom 1974) and the large number of reductive features characteristic of osmeroids.

Begle (1991) and Patterson and Johnson (1995) noted the presence of nuptial tubercles in the osmeroids. According to the data matrix of Begle (p. 38) they are present in *Retropinna*, *Stokellia* and all extant osmerid genera. Tubercles are also present in the fossil osmerid *Speirsaenigma lindoei*. (Wilson and Williams 1991). McDowall (1971a, p. 46) stated that tubercles are present in *Lovettia*, and Begle (1991, p. 39) apparently supported this finding. It seems that Begle inadvertently miscoded *Lovettia* for this character. Begle tentatively suggested that nuptial tubercles have been independently derived on three occasions: in osmerids, retropinnids and *Lovettia*. Alternatively, multiple losses may have occurred. Patterson and Johnson (1995) described this character as ambiguous.

As with the molecular analyses, some of the morphological findings merely reinforce support for universally accepted clades such as *Paragalaxias*. However, other findings may be more controversial. On morphological evidence, the genus *Galaxias* is presently not monophyletic. The Tasmanian mudfish, *G. cleaveri*, is the sister taxon of the New Zealand mudfish genus *Neochanna*. Moreover, *G. zebratus* (South Africa) is apparently the sister group to the clade comprising *Brachygalaxias* (Chile) and *Galaxiella* (Australia). The presence of *Nesogalaxias neocaledonicus* within the *G. brevipinnis* clade calls into question its validity as a monotypic genus. However, this finding is not well supported by bootstrapping.

It is noteworthy that strongly supported morphological clades were also resolved in the combined molecular analysis (Chapter 2), with varying levels of bootstrap support. In addition, the weakly supported placements of *G. parvus* as the sister of *Paragalaxias* (32%) and *Nesogalaxias* with *G. brevipinnis*-like species (26%) both received high bootstrap support from molecular analyses. The general congruence of the independent morphological and molecular phylogenies suggests that they both reflect organismal phylogeny.

In summary, the cladistic analysis of morphological characters provided strong support for relatively recent clades. As in the molecular phylogeny, basal groupings received low bootstrap values. In fact, most topological differences between the molecular and morphological trees are associated with poorly supported basal clades. The lack of support may be due to the limited number of discrete morphological characters available, and the general lack of morphological specialisation within the Galaxiinae. As McDowall (1969) noted, a large number of galaxiines are morphologically similar.

However, this does not necessarily indicate that these species are phylogenetically similar. It appears that rates of morphological evolution are heterogeneous within the galaxiines. Specifically, morphological evolution appears to have been relatively rapid in ecologically specialised groups such as *Paragalaxias* and mudfish, as indicated by the relatively large number of autapomorphies supporting these clades.

CHAPTER 4

Global Parsimony and Galaxiine Evolution

4.1 Global parsimony

The molecular and morphological data sets were combined in a global parsimony analysis of the Galaxiinae (Fig. 4.1). This "total evidence" approach is thought to enhance the detection of real phylogenetic groups, yielding the strongest estimate of phylogeny (Kluge 1989; de Queiroz *et al.* 1995). The combined data consisted of 876 characters, of which 821 were molecular and 51 were morphological. Species for which morphological data were not available (including *G. olidus*, *G. fasciatus* and *G. argenteus*) were included in the global parsimony analysis with morphological character states coded as missing. Of the 278 phylogenetically informative characters, 230 (83%) were molecular (119 for cytochrome *b*, 111 for 16S rRNA) while 48 (17%) were morphological.

The global parsimony analysis revealed substantial support (Fig. 4.1) for all of the groups that were well supported in the combined molecular analysis (Fig. 2.21). However, that is not to say that the morphological data were completely swamped by the molecular characters. The inclusion of morphological characters resulted in increased levels of support for some clades. For example, [*Brachygalaxias*, *Galaxiella*] received a bootstrap value of 89% from global parsimony as opposed to 62% from mtDNA analysis. Similarly, support for [*G. cleaveri*, *Neochanna*] increased from 51% to 94%, and support for the *G. brevipinnis* clade increased from 84% to 96%. *Galaxias zebratus* was placed as the sister of [*Brachygalaxias*, *Galaxiella*] with bootstrap support of 44% as opposed to 21% in the mtDNA parsimony analysis. However, this is not much higher than the support for the alternative placement of *G. zebratus* as the sister of the mudfish clade (36%). The position of *G. maculatus* as the basal galaxiine received moderate support (54%), similar to the support from parsimony analysis of molecular evidence alone. It should be remembered that distance analysis of the molecular data provided only weak bootstrap support for this placement of *G. maculatus*.

Alternative hypothetical groupings received low levels of support. For instance, the *G. truttaceus* and *G. fasciatus* clades were grouped together in only 2% of the bootstrap replicates. Alternatively, the grouping together of the *G. brevipinnis*, *G. truttaceus* and *G. fasciatus* clades received 12% bootstrap support. The placement of *G. truttaceus* as the basal galaxiine (weakly supported by the morphological analysis) received a bootstrap value of 13%.

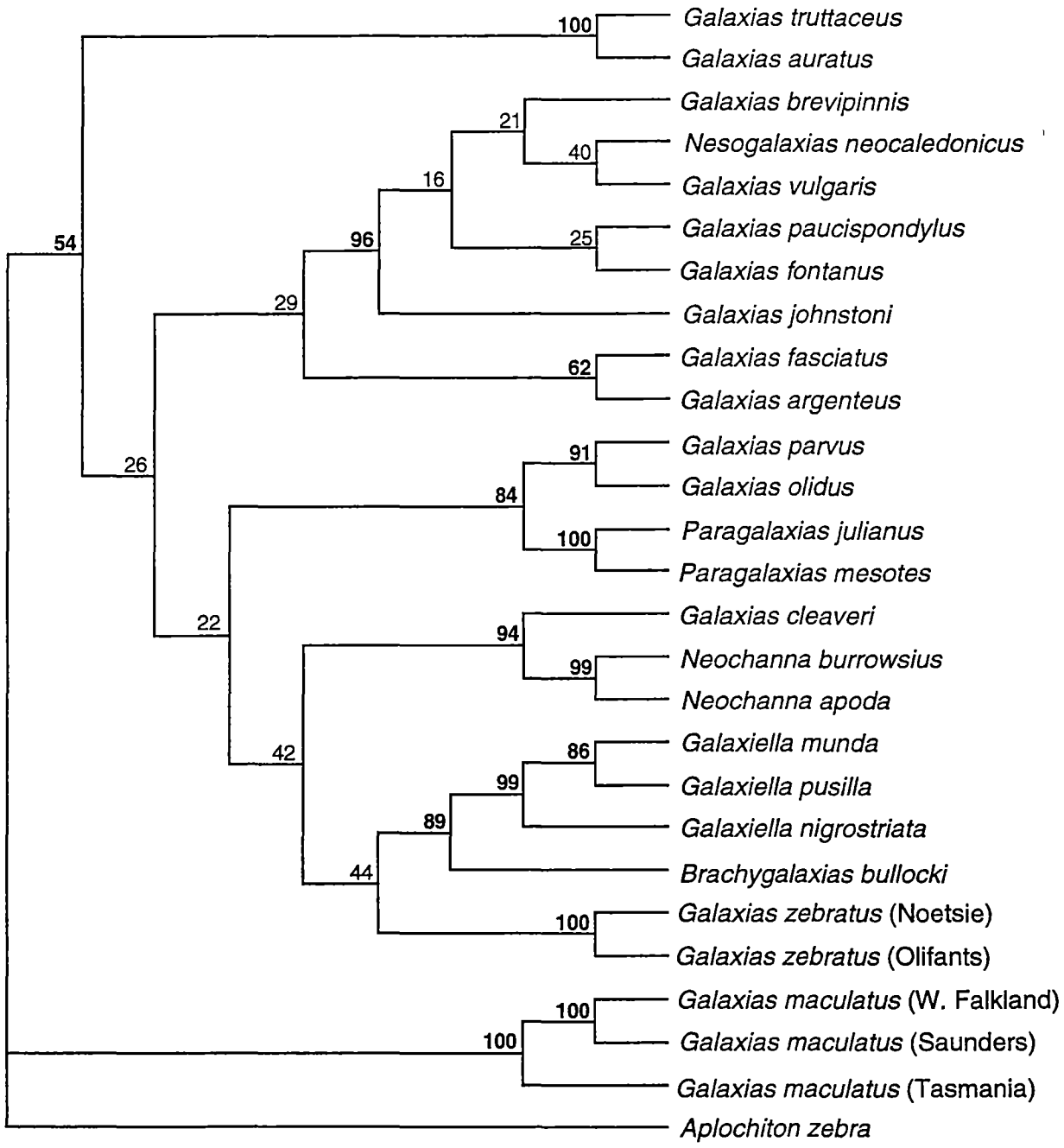

Figure 4.1 Global parsimony analysis of the Galaxiinae based on combined mtDNA sequences and morphological data. Values at branch points indicate bootstrap support as based on 500 resamplings of the data (TV/TI = 2:1); estimates ≥ 50% are shown in bold.

4.2 Recent geological history of the southern continents

The biogeography of the southern continents is reinterpreted as new geological information comes to hand. The currently accepted geological history of the southern continents is as follows. Africa began drifting away from Gondwana at 135 mya, with substantial gaps between southern Africa and Antarctica by 120 mya. However, freshwater connections were still possible between all parts of Gondwana at that time. Even at 100 mya there was still the possibility of freshwater interchange between Africa and South America. Africa was entirely separated from Gondwana by 85 mya (Burrett pers. comm.). New Zealand split from Gondwana about 80 mya (Weissel *et al.* 1977; Kamp 1986) and has been separated from Australia by at least 1,200 km (up to 2,000 km) for the last 60 mya (Cooper and Milliner 1993). Similarly, New Caledonia separated from the northern part of the Lord Howe Rise in the late Cretaceous between 74 and 64 mya with the opening of the New Caledonia Basin (Yan and Kroenke 1993) and has remained well separated from Australia and New Zealand ever since. However, Linder and Crisp (1995) indicated that New Zealand and New Caledonia were connected with each other as recently as 20-30 mya. Similarly, McDowall (1990) and Chazeau (1993) mentioned the possibility of recent connections. There are sea mounts along the Norfolk Ridge which could have emerged in the Miocene during periods of low sea level.

Rosen (1978) indicated that the separation of Australia and South America occurred about 70 mya. This date conflicts with more recent information. Most workers state that Australia and southern South America were connected by Antarctica until about 35-38 mya (Hill pers. comm.). However, ocean temperatures remained warm until 30 mya when the opening of the strait between Australia and Antarctica was great enough to allow an unimpeded circum-polar current (Veevers 1991).

Most biogeographers interpret their data in the light of accepted geological theories. However, when the biogeographical interpretations of some workers conflict with geology, they have sometimes argued that there is a problem with the latter. For example, the cladistic botanists Linder and Crisp (1995) used their biogeographical theories to question well supported geological hypotheses. From a phylogenetic analysis of southern beeches (*Nothofagus*) based on molecular and morphological data, they suggested that Australian taxa have a recent relationship with New Zealand species, closer than either have with South American species. They argued against dispersal as an explanation for this finding. Instead, they proposed alternative geological hypotheses, including land connections between Australia and New Zealand during the Tertiary. There is, however, no geological evidence to suggest that even

discontinuous land links have connected Australia and New Zealand since 80 mya (Cooper and Milliner 1993).

New Zealand is thought to have had extensive marine transgressions in the past, limiting the amount of available freshwater habitat. For example, in the mid-Oligocene, 82% of New Zealand's present land-area was probably submerged (Cooper and Milliner 1993). Pole (1994) suggested that perhaps New Zealand went "completely under" in the Oligocene, eliminating any remnant Gondwanan biota. Thus the biota of New Zealand may be equivalent to that of an oceanic island. He argued that the present flora of New Zealand can be totally attributed to long distance dispersal rather than vicariance. Even the podocarps and southern beeches, which are usually interpreted as Gondwanan, are probably present in New Zealand as a result of dispersal (Hill and Jordan 1993; Pole 1994).

Like New Zealand, New Caledonia has had an unstable geological history. In the Eocene there were drastic changes in the freshwater biota of New Caledonia, related to the extensive overthrust of ultramafic rocks (Chazeau 1993, pers. comm.). The unique elements of New Caledonia's flora are apparently restricted to these ultramafic substrates (Pole 1994). In addition, major marine transgressions occurred during the Eocene and Miocene, submerging most of the present land (Vitt 1995).

4.3 Molecular clock calibrations, phylogeny and biogeography

Molecular clocks can be used to test phylogenetic and biogeographical hypotheses. For example, Taylor and Dodson (1994) estimated that the osmerid genera *Mallotus* and *Osmerus* diverged 4-5.4 mya based on mtDNA. This date conflicts with the suggestion that *Mallotus* is primitive to the fossil *Speirsaenigma* and thus diverged from other osmerids well before the middle of the Paleocene (Wilson and Williams 1991). Unless the mtDNA of osmerids evolves an order of magnitude more slowly than the mtDNA of other fishes, the phylogeny of Wilson and Williams (1991) must be incorrect.

In the current study, three molecular clock calibrations were used to estimate the divergence times of phylogenetic splits within the Galaxiinae (Table. 4.1). Divergence estimates were based solely on the cytochrome *b* data because no clock calibrations are presently available for fish 16S rRNA sequences. Initially, the calibrated divergence rate for cytochrome *b* in *Gasterosteus* was used (2.8% per million years; Orti *et al.* 1994). Alternatively, some workers have used only third codon positions to calibrate a molecular clock. For this study, the silent divergence rate estimated for mammals (10% per million years; Irwin *et al.* 1991) and the silent rate calibrated for *Gasterosteus* (7.4%-8.5% per million years; Orti *et al.* 1994) were used. While these molecular clock

Table 4.1 Divergence times for phylogenetic splits within the Galaxiinae as based on cytochrome *b* data. Three molecular clock calibrations were used: (a) 2.8% cytochrome *b* divergence per million years (fish; Orti *et al.* 1994); (b) 7.4-8.5% cytochrome *b* silent divergence per million years (fish; Orti *et al.* 1994); (c) 10% cytochrome *b* silent divergence per million years (mammals; Irwin *et al.* 1991).

Phylogenetic Split	Cyt b divergence	Silent divergence	Cyt b 2.8%/my	Silent (7.4-8.5%)/my	Silent 10%/my
G. zebratus (Olifants-Noetsie)	13 2%	43.5%	4.7 mya	5.1-5 9 mya	4 4 mya
G. maculatus (Tasmania-Falklands)	18 8%	70 1%	6.7 mya	8 2-9.5 mya	7 0 mya
G maculatus (W. Falkland-Saunders)	4.1%	11.0%	1 5 mya	1.3-1.5 mya	1.1 mya
G. truttaceus-G. auratus	2.1%	6 5%	0.8 mya	0.8-0 9 mya	0.7mya
G. fasciatus-G. argenteus	13.7%	44.7%	4.9 mya	5.3-6 0 mya	4.5 mya
G. johnstoni-G brevipinnis	5.9%	18.2%	2.1 mya	2.1-2 5 mya	1 8 mya
G. vulgaris-G brevipinnis	8 8%	32 3%	3.1 mya	3.8-4.4 mya	3 2 mya
G. fontanus-G brevipinnis	13 8%	47.8%	4.9 mya	5.6-6.5 mya	4.8 mya
G. paucispondylus-G. brevipinnis	10.7%	38.8%	3 8 mya	4.6-5.2 mya	3 9 mya
N. neocaledonicus-G. brevipinnis	8.8%	30.8%	3.1 mya	3.6-4.2 mya	3.1 mya
G. olidus-G. parvus	14.6%	42.9%	5.2 mya	5 0-5 8 mya	4.3 mya
P. julianus-P. mesotes	7 6%	25.7%	2.7 mya	3.0-3.5 mya	2.6 mya
Paragalaxias-[G. olidus, G. parvus]	17.3%	55.2%	6.2 mya	6.5-7.5 mya	5 5 mya
N. apoda-N. burrowsius	13 6%	42 9%	4.9 mya	5 0-5.8 mya	4 3 mya
G. cleaveri-Neochanna	18.1%	68.9%	6 5 mya	8.1-9.3 mya	6 9 mya
G. munda-G. pusilla	14.5%	44.7%	5.2 mya	5.3-6.0 mya	4 5 mya
G. nigrostriata-[G. munda, G pusilla]	18 9%	59.2%	6.8 mya	7.0-7.9 mya	5.9 mya
Galaxiella-Brachygalaxias	26.0%	>100%	9.3 mya	>11.8 mya	>10.0 mya

calibrations are somewhat crude, they provide independent estimates for divergence times within the Galaxiinae. Independent estimates are important because rates of molecular evolution may vary between taxonomic groups (e.g. Martin *et al.* 1992).

The estimation of divergence times was restricted to splits within clades that were strongly supported in both the combined molecular analysis (Fig. 2.21) and the global parsimony analysis (Fig. 4.1). All estimated divergence times were within the last 10 million years (Pliocene or more recent), with the exception of the *Brachygalaxias-Galaxiella* split. The timings of these splits range from 0.7-0.9 mya for *G. truttaceus-G. auratus*, to 6.7-9.5 mya between Tasmanian and Falklands *G. maculatus*. The fact that the mean corrected silent divergence between *Brachygalaxias* and *Galaxiella* cytochrome *b* is over 100% suggests that these sequences are approaching or have reached saturation. Divergence times for saturated sequences cannot be estimated with any degree of confidence. In fact, it has been noted that cytochrome *b* sequences diverge very slowly after about 10-20 million years of separation (Avise *et al.* 1994), confounding estimates of ancient divergence times. It is obvious that the silent rate for mammals (10% per million years) can only be used to estimate divergences that occurred less than 10 mya.

Galaxias zebratus populations

The well supported topology of the cytochrome *b* gene tree (Fig. 2.2) matches the distribution of the sample sites, reflecting increasing isolation by distance (Fig. 4.2). Similarly, McDowall (1973b) found differences in vertebral counts corresponding with geographic distribution. Specifically, eastern populations of *G. zebratus* (from "George") had relatively high vertebral counts (41) whereas specimens from the Olifants River had 38 vertebrae. His statement (p.99) that "south-eastern populations...have fewer vertebrae than the western (Eerste) and northern (Olifants) populations" is incorrect; the reverse is true (McDowall, pers. comm.). This aspect requires re-examination in light of the new distribution records (Cambray *et al.* 1995).

There are several possible explanations for the geographic range (600 km) of the Cape galaxias. These include marine dispersal, transplantation by man, river capture, and eustatic sea level fluctuation. It seems reasonable to preclude the first hypothesis as there is no evidence for diadromy in *G. zebratus*, and substantial genetic divergence between fish from relatively close drainages such as the Noetsie and the Krom Rivers. Similarly, transplantation is unlikely as this species has little value to anglers. River capture is recognised as an important source of gene flow for freshwater limited species (Waters *et al.* 1994; Hynes *et al.* 1996). Within the Cape Fold mountains, drainage evolution may have played a major role in the distribution of fishes (Barnard 1943). Also, there is the possibility that connections between adjacent rivers during periods of eustatic sea level change could have aided dispersion of *G. zebratus*.

The similarity of the Krom and Kouga genotypes suggests that intrapopulation diversity may be low in *G. zebratus*. In addition, it indicates that relatively recent gene flow has occurred between the Krom and Kouga populations. This finding lends no support to the suggestion that these populations are relicts of a Paleocene river capture event (Cambray *et al.* 1995). Given the adjacent nature of the Krom and Kouga drainages (Fig. 4.2), it seems likely that they merged at times of lower sea levels during one or more recent glaciations. However, recent river capture should not be discounted as an alternative explanation. Similarly, I am reticent to choose between river capture and sea level fluctuation when explaining the geographic distribution of the species as a whole.

The high level of genetic divergence observed between most of the populations suggests that *G. zebratus* may represent a species complex rather than a single species. Furthermore, it is possible that distinct genetic types may occur sympatrically, as recently reported in other non-diadromous galaxiines (Watts *et al.* 1995; Allibone *et al.* 1996). It is recommended that another morphological/osteological revision be conducted in parallel with a large-scale analysis of the genetic diversity both within and

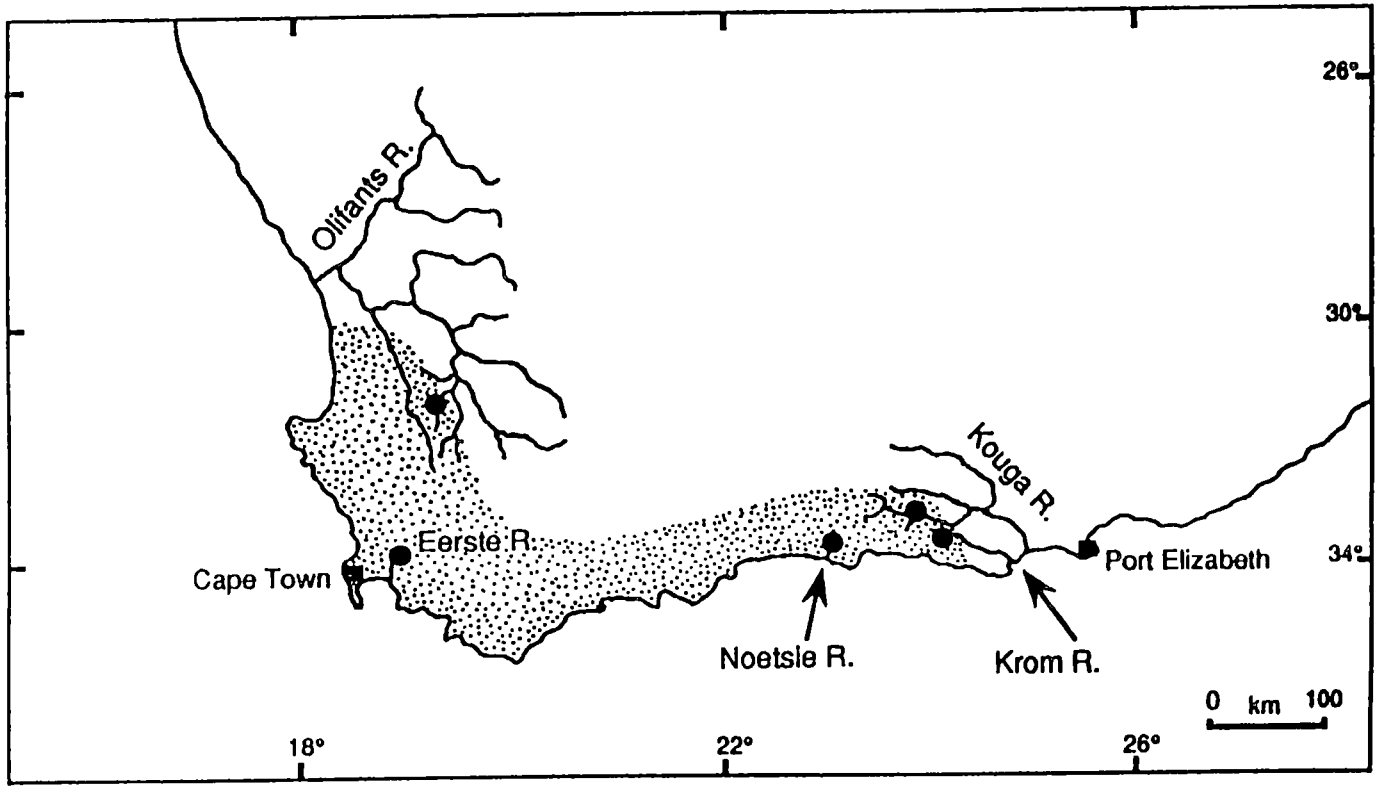

Figure 4.2 Distribution of the South African *Galaxias zebratus* (stippled area), showing the five populations sampled for mtDNA sequencing (closed circles).

between populations. This revision should now include the recently discovered *G. zebratus* from the Krom and Gamtoos (Kouga) River systems.

Galaxias maculatus populations

The taxonomy and biogeography of the widespread *G. maculatus* have long been controversial. A number of described species were synonymised as *G. maculatus* by McDowall (1967). He regarded different populations as nearly indistinguishable. The range of this species includes Australia, New Zealand, South America, and the Falkland Islands (including many locations on both East and West Falkland; McDowall 1971b). Rosen (1974, 1978) and others argued that the distribution of *G. maculatus* is best explained by continental drift and a subsequent slow rate of morphological evolution. In contrast, McDowall (1971b, 1978b, 1990) has maintained that the wide distribution of this species represents oceanic dispersal.

Recently, Berra *et al.* (1996) conducted an allozyme electrophoresis study of *G. maculatus* populations from Western Australia, New South Wales, New Zealand and Chile. The analysis was based on six presumptive loci, four of which were used to screen only five individuals from each population. They reported only minor genetic differentiation between these populations and concluded that trans-Pacific geneflow is ongoing. However, the low genetic distance (0.014) and high genetic identity (0.987) reported between populations from eastern and western Australia appear to conflict with the findings of Pavuk (unpublished data). She examined the population genetic structure of *G. maculatus* with six presumptive loci, surveying about 800 specimens from a total of nine populations representing Western Australia, Victoria, Tasmania and New Zealand. The minimum sample size was 48. Pavuk reported a significant level of genetic distance (range 0.094-0.117) and low genetic identity (range 0.890-0.911) between the Western Australian and other populations. She concluded that the Western Australian gene pool was clearly isolated from that of eastern Australian and New Zealand *G. maculatus*.

Molecular clock calibrations (Table 4.1) suggest that the Tasmanian and Falkland Island *G. maculatus* examined in this study have been genetically isolated since 6.7-9.5 mya. This finding further questions the conclusion of Berra *et al.* (1996) that inter-populational genetic divergence is minor for this species. Unfortunately, no *G. maculatus* from Chile were obtained for the present study. However, given the geographic distances involved, it would be reasonable to expect Falkland and Chilean *G. maculatus* to be more closely related to each other than either is to Tasmanian specimens. Presumably, only a major zoogeographical barrier (e.g. unfavourable ocean currents) would prevent this. It seems relatively likely that the data of Berra *et al.*

(1996) significantly underestimate the level of genetic divergence between *G. maculatus* populations from either side of the Pacific Ocean. The findings of Pavuk (see above) lend additional support to this hypothesis.

The high level of genetic divergence between Tasmanian and Falkland *G. maculatus* indicates that gene flow between these populations has long ceased. However, the estimated divergence time for the cytochrome *b* sequences postdates the presently accepted timing of the South America-Australia vicariance by at least 20 million years. Thus, it seems that oceanic dispersal is the most likely explanation for the wide distribution of *G. maculatus*. However, the dispersal powers of some populations of this species are apparently far more limited than has been suggested by Berra *et al.* (1996) and others. In fact, *G. maculatus* may represent a species complex.

According to the cytochrome *b* data, it seems that the West Falkland and Saunders Island genotypes diverged at least one million years before present (range 1.1-1.5 mya), predating the estimated splits of some closely related galaxiine species. This is surprising given that they are supposedly diadromous and geographically separated by less than 60 km. Indeed, the larvae of *G. maculatus* spend up to six months in the sea (McDowall *et al.* 1994) and have been caught 700 km from land (McDowall *et al.* 1975). It may be that one of these populations is landlocked, but this is speculative as there are no life history data for Falkland *G. maculatus*. Superficially, the West Falkland specimens closely resemble typical *G. maculatus*. In contrast, specimens from Saunders Island are much more strongly pigmented, and appear stouter than the West Falkland *G. maculatus*. It is possible that these superficial differences reflect preservation differences, but this is unlikely as both samples were stored in 90% ethanol. A thorough morphological revision may be required to resolve the specific status of the Falkland *G. maculatus*.

Galaxias truttaceus-G. auratus

Ovenden *et al.* (1993) stated that *G. auratus* was "probably derived from a landlocked population of the diadromous *G. truttaceus*". Similar speciation is presently under way in more recently landlocked *G. truttaceus* (Ovenden and White 1990). Lakes Sorell and Crescent are thought to predate recent glaciations (Davies 1974), but are probably less than 100,000 years old (Banks pers. comm.). However, the estimated timing of the *G. truttaceus-G. auratus* split (about 0.7-0.9 mya) predates the hypothesised lake formation by at least 600,000 years. This may reflect genetic divergence (and perhaps speciation) prior to the formation of the lakes. The sequence divergences reported between *G. truttaceus* and *G. auratus* (2.1% for cytochrome *b*; 1.0% for 16S rRNA) are roughly similar to the value estimated for the entire mitochondrial genome based on

RFLP data for these species (1.9%, Ovenden *et al.* 1993). Ovenden *et al.* (1993) noted that mtDNA divergence was very high relative to the nuclear DNA divergence between these species. They suggested that this phenomenon may reflect the "abrupt and permanent effect of bottlenecks upon the intraspecific mtDNA diversity of *G. auratus*...in their confined lake habitat".

Galaxias fasciatus-G. argenteus

According to cytochrome *b* data, *G. fasciatus* and *G. argenteus* diverged about 4.5-6.0 mya. This is close to the timing of the *N. apoda -N. burrowsius* separation, and may be related to the Tertiary uplift of New Zealand's Southern Alps. However, such uplift would not have created reproductive barriers between these diadromous taxa. Alternatively, *G. fasciatus* and *G. argenteus* may represent independent colonisations by a diadromous Australian ancestor. If marine dispersal is (or was) sporadic, as suggested by the *G. maculatus* data, speciation may have rapidly followed such colonisation. Unfortunately, a lack of information precludes further speculation on this topic. The higher affinities of *G. fasciatus* and *G. argenteus* are still not certain. Although the present study provides little support for the suggested relationship between these species and *G. truttaceus* (McDowall 1990), such an affinity cannot be ruled out. Furthermore, recent fossil findings may indicate the presence of "large" galaxiines in New Zealand during the early Miocene (>20 mya, Pole pers. comm.), well before the split of *G. fasciatus* and *G. argenteus*. It is recommended that *G. postvectis* be included in future studies, given its apparent morphological (dark fin margins; McDowall 1990) and karyological similarities (Bennett 1984) to *G. truttaceus*.

Galaxias brevipinnis-G. vulgaris-G. fontanus-G. johnstoni-G. paucispondylus-Nesogalaxias

The analysis of combined molecular and morphological data provide very strong support for a close phylogenetic relationship between these species. However, no clear phylogenetic relationships were identified within this clade, making the estimation of divergence times problematic. The lack of resolution may be a result of a number of factors, including rapid cladogenesis and stochastic lineage sorting (see Chapter 2). It has been hypothesised that *G. vulgaris* arose from one or more landlocked populations of the diadromous *G. brevipinnis* (McDowall 1990; Allibone and Wallis 1993). Similarly, a close phylogenetic relationship was suggested between *G. fontanus*, *G. johnstoni* and *G. brevipinnis* (Fulton 1978; Johnson *et al.* 1981). I hypothesise that *G. brevipinnis* (or a *G. brevipinnis*-like ancestor) was the precursor for all non-diadromous species in this clade. If this is the case, the estimated divergence times are 1.8-2.5 mya for the *G. johnstoni-G. brevipinnis* split, 3.1-4.4 mya for the *G. brevipinnis-G. vulgaris* split, and 4.8-6.5 mya for *G. fontanus-G. brevipinnis*.

McDowall (1969, 1970, 1990) considered that the New Zealand taxa *G. paucispondylus*, *G. divergens* and *G. prognathus* represent a distinct "alpine galaxiid" clade of unknown origin. It is likely that the inclusion of *G. divergens* and *G. prognathus* in the present study would support the alpine clade and place it within the large *G. brevipinnis* group. McDowall (1990) argued that *G. paucispondylus* is the least specialised of the alpine galaxiines. He suggested that the common ancestor of the alpine galaxiines was once widespread in New Zealand, perhaps before the uplift of the Southern Alps 3-5 mya. The cytochrome *b* data lend some support to this hypothesis, indicating that *G. paucispondylus* diverged from a *G. brevipinnis*-like ancestor 3.9-5.2 mya, possibly just preceeding the southern uplift.

The biogeographical affinities of the New Caledonian galaxiine, a geographic outlier, have generated considerable interest. McDowall (1968) suggested that this species colonised New Caledonia by marine dispersal during a cool period of the Pliocene or Pleistocene, subsequent warming confining it to upland lakes. He considered that Australia was the most likely source for such dispersal. Subsequently, McDowall (1990) suggested that *N. neocaledonicus* may indicate a recent connection between New Zealand and New Caledonia. The mtDNA sequence data presented in the current study indicate that *N. neocaledonicus* diverged from *G. brevipinnis* 3.1-4.2 mya. Unless land/island links between New Zealand and New Caledonia were present as recently as the late Pliocene, marine dispersal is the most likely explanation for the origin of *N. neocaledonicus*.

Galaxias parvus-G. olidus

Until the current study, no worker has suggested that *G. parvus and G. olidus* share a close phyletic relationship. These species were paired by cluster analyses of morphometric and meristic data (Johnson *et al.* 1983) but this unexpected result was interpreted as reflecting convergence rather than phylogenetic relationship. However, the findings of the present study suggest that the opposite is true. It may be that biogeographical implications have prevented serious consideration of such a phylogenetic relationship by past workers. Tasmanian *G. parvus* and Victorian *G. olidus* apparently diverged from a common ancestor about 4.3-5.8 mya (late Pliocene). The ancestor of these species may have occupied the drainages of south-eastern Australia and Tasmania during cooler periods. Subsequent warming or interspecific competition may have isolated populations and resulted in speciation. Alternatively, *G. parvus* and *G. olidus* may be landlocked derivatives of an extinct diadromous ancestor. Given the wide distribution of *G. olidus* and the possibility of divergent populations or

cryptic species (e.g. *fuscus* "variety", Allen 1989), additional representatives of this species should be included in future genetic studies of the Galaxiinae.

Paragalaxias mesotes-P. julianus

According to cytochrome *b* data, *P. mesotes* and *P. julianus* split between 2.6 and 3.5 mya. This supports the suggestion of McDowall and Fulton (1978) that *Paragalaxias* predates the Pleistocene (>2 mya). *Paragalaxias julianus* occurs in south-flowing drainages, while *P. mesotes* is restricted to a north-flowing drainage of the Tasmanian central plateau. The distribution of *Paragalaxias* mirrors the distribution of the endemic syncarid *Paranaspides* (Fulton 1982). It has been suggested that this shared distribution pattern was caused by river capture (McDowall and Fulton 1978). Thus, the timing of the *P. mesotes-P. julianus* split (2.6-3.5 mya) may represent an approximate date for the hypothesised change in drainage pattern.

Paragalaxias-Galaxias olidus-G. parvus

McDowall and Fulton (1978) noted that "the primitive stock from which *Paragalaxias* is derived is not yet evident". They suggested that this genus could be derived from an aplochitonid by the loss of the adipose fin or, more likely, derived from a galaxiine by the forward movement of the dorsal fin. The findings of the present study indicate that the latter hypothesis is correct. Specifically, mtDNA sequences indicate that *Paragalaxias* is closely related to the *G. olidus-G. parvus* clade. The two groups apparently diverged in the Pliocene, up to about 7.5 mya. On this evidence, it is clear that *Paragalaxias* has exhibited a relatively high rate of morphological evolution in comparison to other galaxiines. Derived characteristics such as a long head, long vomerine shaft, forward dorsal fin and short prepelvic length have evolved in a time span during which other taxa have undergone virtually no change. For example Tasmanian and Falkland *G. maculatus* are apparently conspecific after up to 9.5 million years of isolation. Similarly, fossil galaxiines from 7-8 mya are assignable to extant species (McDowall 1990).

Neochanna-Galaxias cleaveri

The estimated timing of the *N. apoda-N. burrowsius* split (4.3-5.8 mya) is consistent with the recent geological history of New Zealand. McDowall (1990) views the Tertiary uplift of New Zealand's Southern Alps 3-5 mya (Fleming 1979) as a vicariant event which gave rise to the disjunct distribution of these species.

There are two likely explanations for the presence of related mudfish on either side of the Tasman sea. The first hypothesis involves an ancient Gondwanan origin for the mudfishes with subsequent continental drift, as would be favoured by Rosen (1974,

1978), Croizat *et al.* (1974) and Craw (1979). The second possibility is marine dispersal as generally favoured by McDowall (1978b, 1990).

Acceptance of the vicariant hypothesis implies that the phylogenetic split occurred about 80 mya. Given the relatively recent divergence times estimated for *G. cleaveri* and New Zealand mudfish cytochrome *b* (6.5-9.3 mya), there would seem to be little support for a Gondwanan origin for this clade. Furthermore, the fact that the Tasmanian mudfish has a marine larval stage, and that trans-Tasman currents flow eastward lend support to the hypothesis that the mudfish had an Australian origin and colonised New Zealand as a result of marine dispersal. The alternative possibility that the mudfish group arose in New Zealand and subsequently colonised Australia is unlikely given the eastward direction of air and water movement.

Galaxiella

McDowall (1978a) suggested that, of the three *Galaxiella* species, *G. pusilla* and *G. nigrostriata* are the most similar. He noted that *G. munda* has a shallower body, higher vertebral counts, fewer pectoral rays, and more pelvic rays than *G. pusilla* and *G. nigrostriata*. McDowall hypothesised that *G. munda* is a neotenic derivative of either *G. pusilla* and *G. nigrostriata*. Berra and Allen (1989) stated that the Western Australian *G. munda* and *G. nigrostriata* are highly similar, noting that none of McDowall's (1978a) characters are completely diagnostic for these sympatric species. The findings of the current study indicate that it is *G. munda* and *G. pusilla* which are most closely related, having split 4.5-6.0 mya. The common ancestor of these species apparently diverged from *G. nigrostriata* up to 7.9 mya.

The presence of *G. pusilla* on either side of Bass Strait is most easily explained by land bridges during Pleistocene glaciations. It is also likely that dispersal between eastern and western Australia was more possible during periods of low sea-level. It is probable that *Galaxiella* was originally present in Western Australia. A *G. nigrostriata* like ancestor may have initially been confined to isolated water bodies, with little dispersal ability, as suggested by the typical habitat and restricted distribution of this species (McDowall and Frankenberg 1981; Pen *et al.* 1993). About 6-8 mya, this species may have split into *G. nigrostriata* and *G. munda* as a result of an ecological shift away from temporary water bodies and early spawning (Pen *et al.* 1993) towards flowing water and later spawning. Alternatively, the original *Galaxiella* may have been similar to *G. munda*, and *G. nigrostriata* may have arisen 6-8 mya due to ecological specialisation. Given its greater vagility, the *G. munda*-like ancestor may have dispersed during a period of low sea level to occupy both southeast and southwest Australia. Subsequently, higher sea levels may have resulted in reproductive isolation, giving rise

to *G. munda* (southwest Australia) and *G. pusilla* (southeast Australia) about 4.5-6 mya.

However, the above explanations of *Galaxiella* biogeography are largely speculative and must be treated with caution. In addition, current distributions may be poor indicators of past distributions. For example, the range of these species may have extended further north during glacial periods. Moreover, a single fossil may indicate that a group was once present in a region where it is now extinct (Wilson and Williams 1993). Because this genus is non-diadromous, it may contain unforeseen levels of intraspecific genetic diversity or cryptic species. An extensive population and evolutionary genetic study of *Galaxiella* is warranted.

Brachygalaxias-Galaxiella

The presence of members of this clade in both Australia and South America could be explained in terms of either dispersal or vicariance biogeography. Both *Brachygalaxias* and *Galaxiella* contain only non-diadromous species. Given the absence of *Brachygalaxias* and *Galaxiella* from New Zealand, and the apparent absence of diadromy, there is no reason to invoke oceanic dispersal for this clade. In contrast, it is known that Australia and South America were connected by Antarctica as recently as 35-38 mya. Vicariance is therefore the most plausible explanation for the distribution of *Brachygalaxias* and *Galaxiella*. The molecular data do not conflict with this hypothesis. While the timing of the *Brachygalaxias-Galaxiella* divergence is confounded by saturation of cytochrome *b* sequences, the phylogenetic split almost certainly exceeds 10 mya and may be considerably older.

On the data presented in this study, *Galaxias zebratus* may be the closest common ancestor of the *Brachygalaxias-Galaxiella* clade. Like the Australian and South American species, the South African galaxiine is freshwater-limited. McDowall (1973c) admitted that *Brachygalaxias*, *Galaxiella*, and *G. zebratus* "may form a very ancient gondwanian relict group". The minimum age of this hypothetical clade would be about 85 million years. A considerable amount of highly conserved nucleotide sequence data (probably nuclear DNA) is required to test this hypothesis.

Summary

In summary, I hypothesise that the galaxiines arose from an *Aplochiton*-like ancestor before the rifting of the southern continents 85 mya. The first galaxiine was probably a diadromous species, possibly similar to *G. maculatus*. Subsequent radiations within the Galaxiinae fall into three general biogeographical categories. Firstly, clades of non-diadromous species that are limited to a single landmass probably attained their current

distribution as a result of changing drainage patterns through river capture or sea-level fluctuation. Such clades include *Galaxiella*, *Paragalaxias*, *Neochanna* and divergent populations of *G. zebratus*. Secondly, some clades include diadromous species and are represented on more than one landmass, but postdate continental vicariance. Such groups include the mudfish and *G. brevipinnis* clades, and populations of *G. maculatus* and are best explained by oceanic dispersal. Finally, one non-diadromous clade is represented on more than one landmass and is best explained by ancient continental vicariance. Specifically, *Galaxiella*, *Brachygalaxias* (and possibly *G. zebratus*) are likely to represent an ancient Gondwanan radiation. The phylogenetic and biogeographical affinities of some taxa not included in the present study remain uncertain. Such species include *G. rekohua* (Chatham Islands), *G. postvectis* (New Zealand), *G. globiceps* and *G. platei* (South America). It is recommended that these taxa be included in future phylogenetic studies of the Galaxiinae.

4.4 Taxonomic recommendations

Traditionally, taxonomic decisions have tended to ignore evolutionary relationships. For example, Linnaean systematists link crocodiles with lizards, despite the commonly accepted notion that crocodiles are phylogenetically closer to birds (Ridley 1985). In contrast, evolutionary systematists argue that classification should be based on phylogeny. Phylogenetic taxonomy aims to "represent relationships of common descent using a system of names" (de Queiroz and Gauthier 1992). However, the translation of a cladogram into a cladistic classification involves a number of subjective decisions about higher groupings (Carpenter 1993). Phylogenetic relationships are best elucidated with a combination of both molecular and morphological data (Finnerty and Block 1994b). McDowall (1973d) was hesitant to make taxonomic decisions that might create "zoogeographic problems". However, if galaxiine taxonomy is to adequately represent galaxiine phylogeny, as revealed by independent molecular and morphological analyses and global parsimony, the taxonomic status of some species must be reconsidered. The discussion below is limited to clades that are strongly supported by global parsimony. While the generic positions of *G. maculatus* and *G. zebratus* are apparently unsatisfactory, they will not be discussed any further because the phylogenetic affinities of these species are yet to be conclusively resolved.

Taxonomy of mudfish

McDowall (1990) was equivocal about the affinities of the Tasmanian mudfish, suggesting that it may be related to *Neochanna* or alternatively may represent convergent evolution. However, the global parsimony analysis provides unequivocal bootstrap support (94%) for a close phylogenetic relationship between *G. cleaveri* and *Neochanna*. This repudiates the statement of Andrews (1973) that the morphological

and behavioural similarities of the Tasmanian and New Zealand mudfish are a result of parallel evolution. While relatively few of the behavioural and morphological features shared by mudfish species are unique within the Galaxiinae, parsimony indicates that the similarities reflect a common ancestry. Specifically, mudfish are united by their aestivating ability, rounded caudal fin with strong flanges, small eyes, Y-shaped vomer, strong palatine spurs, reduced or absent pelvic fins and mesopterygoidal teeth, absence of median ethmoid ossification, and features of the supraorbital canal. The Tasmanian mudfish is included as the least specialised member of the genus *Neochanna*.

Taxonomy of Brachygalaxias-Galaxiella

The molecular and morphological analyses independently unite the monotypic South American genus *Brachygalaxias* with the Australian genus *Galaxiella*. The high bootstrap value (89%) obtained from the global parsimony analysis indicates that this grouping is well-supported. Originally, the Australian and South American species were all included in *Brachygalaxias*. However, McDowall (1973c) limited the genus to contain only the South American species because it has an extended premaxillary alveolar process, a character that is phylogenetically uninformative. McDowall (1973d, 1978a) noted similarities between *Galaxiella* and *Brachygalaxias*, but claimed that there was little to indicate a phylogenetic relationship. He also suggested that the phylogenetic and zoogeographical affinities of these fishes would always remain unanswered. Times change, however, and it is now clear that his suggestion that the inclusion of all four species in one genus "confuses the understanding of relationships" (McDowall 1984) is incorrect. In fact the reverse is true. McDowall's placement of these taxa in separate genera ignores evolutionary relationships and implies that their similarities represent convergence rather than common inheritance. The orange stripe, posterior dorsal fin, large eyes, and reduced supraneural series common to these species are unique within the Galaxiinae. Furthermore, mtDNA sequences and additional morphological characters reinforce the common ancestry of *Galaxiella* and *Brachygalaxias*. The Australian species are therefore revised to *Brachygalaxias*, with *Galaxiella* used for subgeneric ranking if necessary.

Taxonomy of Nesogalaxias neocaledonicus

McDowall noted that *N. neocaledonicus* was superficially similar to some members of the genus *Galaxias*. However, McDowall (1968, 1969) placed the New Caledonian galaxiine in a separate genus on the basis of the absence of pleural ribs posterior to the pelvic girdle. A few other characters were also mentioned as having generic significance, including the absence of epipleurals and postcleithra. However, both Frankenberg (1969) and the current study found epipleurals to be present in

Nesogalaxias. Furthermore, despite noting that postcleithra are also absent in *G. paucispondylus*, McDowall (1968) dismissed this species as unrelated to *N. neocaledonicus*. Such an assertion is surprising given that he went on to admit that the affinities of *Nesogalaxias* were "quite unknown". The present study concludes that *Nesogalaxias* is a member of a clade comprising *G. brevipinnis* and other closely related species of *Galaxias*, including *G. paucispondylus*. The generic status of the New Caledonian galaxiine is a subjective decision concerning the relative importance of phylogeny and a single phylogenetically uninformative morphological character. Given the trend towards phylogenetic taxonomy, and the general similarities and close phylogenetic relationship of *Nesogalaxias* and species such as *G. brevipinnis* (split 3-4 mya), it could be concluded that the New Caledonian species belongs in *Galaxias*. Alternatively, its placement in a monotypic genus could be upheld as it is the only tropical galaxiine, and a species which has undergone a considerable amount of morphological evolution in a relatively short time.

Taxonomy of Galaxias olidus and G. parvus

Unfortunately, no specimens of *G. olidus* were included in the current morphological analysis of the Galaxiinae. Thus the strong support (91%) for [*G. olidus*, *G. parvus*] is based on molecular data only. However, when the genetic similarity is coupled with the apparent morphological similarity of these species (Johnson *et al.* 1983), there can be little doubt that *G. olidus* and *G. parvus* represent a real phylogenetic clade. With limited morphological data, the generic status of these species is problematic. The mtDNA phylogeny suggests that they should be allocated to *Paragalaxias*, but morphology (in the case of *G. parvus*) does not. Alternatively, *G. olidus* and *G. parvus* may belong in a new genus. Until a thorough osteological examination of *G. olidus* is complete, the generic status of these species should probably remain unchanged.

4.5 Conclusions

The molecular and morphological analyses provide novel insights into galaxiine phylogeny. Both molecular, morphological and global parsimony analyses provide strong support for clades, some of which were previously regarded as questionable because of their biogeographical implications. Molecular data were particularly useful in resolving the relationships of clades that probably diverged in the last 10 million years. The phylogenetic signal increased with more molecular characters, as shown by the higher bootstrap support for clades in the combined cytochrome *b* and 16S rRNA analysis. Molecular clock calibrations provided divergence estimates that seem to be reasonably consistent with current biogeographical theories. The wide distribution of the galaxiines is interpreted as a large number of recent dispersal events superimposed

on relatively few ancient Gondwanan radiations. Furthermore, this study recommends the analysis of linked mtDNA sequences and combined molecular and morphological data (total evidence), as both strategies increase phylogenetic resolution within the Galaxiinae.

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